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(54) Title: METHOD AND REAGENT FOR INHIBITING CANCER DEVELOPMENT

(57) Abstract

An enzymatic RNA molecule which specifically cleaves mRNA encoded by an mdr-1 gene, or associated with development or maintenance of chronic myelogenous leukemia, promyelocytic leukemia, Burkitt's lymphoma or acute lymphocytic leukemia, follicular lymphoma, B-cell acute lymphocytic leukemia, breast cancer, colon carcinoma, neuroblastoma, and lung cancer.

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DESCRIPTION

METHOD AND REAGENT FOR INHIBITING CANCER DEVELOPMENT

Background of the Invention

This invention relates to methods for treating cancer, and in particular, growth of a transformed cell, and inhibition of progression to a transformed phenotype in pre-neoplastic cells.

Transformation is a cumulative process whereby normal control of cell growth and differentiation is interrupted, usually through the accumulation of mutations affecting the expression of genes that regulate cell growth and differentiation.

Scanlon WO91/18625, WO91/18624, and WO91/18913 describes a ribozyme effective to cleave oncogene RNA in the H-ras gene. This ribozyme is said to inhibit C-fos expression in response to cis-plantin or other stimuli. Reddy, WO 92/00080 and U.S. Serial No. 07/544,199 (filed June 26, 1990), describes use of ribozymes as therapeutic agents for leukemias, such as chronic myelogenous leukemia (CML) by targeting specific junction regions of the *bcr-abl* fusion transcript.

Summary of the Invention

This invention concerns use of a ribozyme targeted to the P-glycoprotein (mdr-1 gene) or other cancer-related genes prior to and/or during administration of anticancer chemotherapeutic agents. Inclusion of such a ribozyme increases the susceptibility of the transformed cells to such agents.

Applicant notes that relapse of disease caused by cancerous cells after administration of chemotherapeutic agents is a major problem in obtaining lasting remissions in a clinic. In some neoplasias, relapse is caused by the expansion of a population of transformed cells resistant to the initial and subsequent

forms of chemotherapy due to inappropriate expression of the mdr-1 gene, also called P-glycoprotein. Such expression is usually caused by selection of transformed cells that have amplified the mdr-1 gene and thus produce increased amounts of the mdr-1 gene product. Applicant describes treatment of and prevention of this condition by use of ribozymes targeted to the mRNA encoded by this gene.

The mdr-1 gene encodes a 170 kDa integral membrane transport protein that confers resistance to certain chemotherapeutic agents such as colchicine, doxorubicin, actinomycin D and vinblastine (reviewed in Gottesman and Pastan, 263 J. Biol. Chem. 12163, 1988). The gene has been isolated from both human and rodent cells selected *in vitro* for resistance to such agents (Roninson et al., 309 Nature 626, 1984; and Roninson et al., 83 Proc. Natl. Acad. Sci USA, 4538, 1986), and the entire 4.5-kb *MDR*1 transcript encoding the human *MDR*1 has been sequenced (Chen et al., 47 Cell 381, 1986, EMBL accession # M14758). The gene is normally expressed in the cells of the colon, small intestine, kidney, liver and adrenal gland. High levels of *MDR*1 transcript have been found in adenocarcinomas that are intrinsically resistant to a broad range of chemotherapeutic agents, such as those derived from adrenal, kidney, liver and bowel.

The invention features use of ribozymes to inhibit the development or expression of a transformed phenotype in man and other animals by modulating expression of a gene that either contributes to, or inhibits the expression of CML, promyelocytic leukemia, Burkitt's lymphoma, acute lymphocytic leukemia, follicular lymphoma, B-cell acute lymphocytic leukemia, breast cancer, colon carcinoma, neuroblastoma, lung cancer, and other neoplastic conditions. Cleavage of targeted mRNAs expressed in pre-neoplastic and transformed cells elicits inhibition of the transformed state.

Ribozymes are RNA molecules having an enzymatic activity which is able to repeatedly cleave other separate RNA molecules in a nucleotide base sequence specific manner. Such enzymatic RNA molecules can be targeted to virtually any RNA transcript and efficient cleavage has been achieved *in vitro*. Kim et al., 84 Proc. Natl. Acad. Sci. USA 8788, 1987; Haseloff and Gerlach, 334 Nature 585, 1988; Cech, 260 JAMA 3030, 1988; and Jefferies et al., 17 Nucleic Acids Research 1371, 1989.

Ribozymes act by first binding to a target RNA. Such binding occurs through the target RNA binding portion of a ribozyme which is held in close proximity to an enzymatic portion of the RNA which acts to cleave the target RNA. Thus, the ribozyme first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After a ribozyme has bound and cleaved its RNA target it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

The enzymatic nature of a ribozyme is advantageous over other technologies, such as antisense technology (where a nucleic acid molecule simply binds to a nucleic acid target to block its translation) since the effective concentration of ribozyme necessary to effect a therapeutic treatment is lower than that of an antisense oligonucleotide. This advantage reflects the ability of the ribozyme to act enzymatically. Thus, a single ribozyme molecule is able to cleave many molecules of target RNA. In addition, the ribozyme is a highly specific inhibitor, with the specificity of inhibition depending not only on the base pairing mechanism of binding, but also on the mechanism by which the molecule inhibits the expression of the RNA to which it binds. That is, the inhibition is caused by cleavage of the RNA target and so specificity is defined as the ratio of the

rate of cleavage of the targeted RNA over the rate of cleavage of non-targeted RNA. This cleavage mechanism is dependent upon factors additional to those involved in base pairing. Thus, it is thought that the specificity of action of a ribozyme is greater than that of antisense oligonucleotide binding the same RNA site.

This class of chemicals exhibits a high degree of specificity for cleavage of the intended target mRNA. Consequently, the ribozyme agent will only affect cells expressing that particular gene, and will not be toxic to normal tissues.

Thus, the invention features novel enzymatic RNA molecules, or ribozymes, and methods for their use for inhibiting cancer-related mRNA expression. Such ribozymes can be used in a method for treatment of disease caused by expression of the cancer-related genes in man and other animals, including other primates. This conclusion, as noted above, is based upon the finding that many forms of cancer become unresponsive to certain chemotherapeutic agents as a result of overexpression of, e.g., the mdr-1 gene. The advantage of using ribozymes of the present invention is their ability to specifically cleave the targeted mRNA, ultimately leading to a reduction in target gene activity through a decrease in level of the gene product. Use of mdr-1 specific ribozymes removes the mechanism of drug resistance used by transformed cells, and thus enhances drug therapies for tumor cell growth. These agents can be administered prior to and during chemotherapeutic treatment of those neoplasias known to have a high incidence of drug resistance, or can be used prophylactically for all neoplasias.

The invention can also be used to treat cancer or pre-neoplastic conditions. Two preferred administration protocols can be used, either *in vivo* administration to reduce the tumor burden, or *ex vivo* treatment to eradicate transformed cells from tissues such as bone marrow prior to reimplantation.

Thus, in a first aspect, the invention features an enzymatic RNA molecule (or ribozyme) which cleaves mdr-1 mRNA (i.e., mRNA expressed from the mdr-1 gene), or its equivalent. In particular, the invention features hammerhead ribozymes designed to cleave accessible areas of the mdr-1 mRNA. Such areas include those sequences shown in Fig. 2.

In a second aspect, the invention features an enzymatic RNA molecule (or ribozyme) which cleaves mRNA associated with development or maintenance of CML, promyelocytic leukemia, Burkitt's lymphoma or acute lymphocytic leukemia, follicular lymphoma, B-cell acute lymphocytic leukemia, breast cancer, colon carcinoma, neuroblastoma, and lung cancer, including mRNA targets disclosed in Figs. 3 to 11. Such mRNA is recognized by those in the art to encode an aberrant cellular protein which is able to control cellular proliferation, and is directly linked to (correlated with) the presence of the leukemic phenotype.

By "enzymatic RNA molecule" it is meant an RNA molecule which has complementarity in a substrate binding region to a specified mRNA target, and also has an enzymatic activity which is active to specifically cleave RNA in that mRNA. That is, the enzymatic RNA molecule is able to intermolecularly cleave mRNA and thereby inactivate a target mRNA molecule. This complementarity functions to allow sufficient hybridization of the enzymatic RNA molecule to the target RNA to allow the cleavage to occur. For *in vivo* use, such complementarity may be between 30 and 45 bases. One hundred percent complementarity is preferred, but complementarity as low as 50-75% may also be useful in this invention.

By "equivalent" RNA to mdr-1 mRNA is meant to include those naturally occurring mRNA molecules associated with neoplastic diseases in various animals, including humans, and other primates, which have similar structures and functions to that mdr-1 mRNA in humans.

The deduced sequences of the mouse and human P-glycoproteins are 80% identical.

In preferred embodiments, the enzymatic RNA molecule is formed in a hammerhead motif, but may also be formed in the motif of a hairpin, hepatitis delta virus, group I intron or RNaseP-like RNA (in association with an RNA guide sequence). Examples of such hammerhead motifs are described by Rossi et al., 8 Aids Research and Human Retroviruses 183, 1992; of hairpin motifs by Hampel et al., "RNA Catalyst for Cleaving Specific RNA Sequences", filed September 20, 1989, which is a continuation-in-part of U.S. Serial No. 07/247,100 filed September 20, 1988, Hampel and Tritz, 28 Biochemistry 4929, 1989 and Hampel et al., 18 Nucleic Acids Research 299, 1990; an example of the hepatitis delta virus motif is described by Perrotta and Been, 31 Biochemistry 16, 1992; of the RNaseP motif by Guerrier-Takada et al., 35 Cell 849, 1983; and of the group I intron by Cech et al., U.S. Patent 4,987,071. These specific motifs are not limiting in the invention and those skilled in the art will recognize that all that is important in an enzymatic RNA molecule of this invention is that it has a specific substrate binding site which is complementary to one or more of the target mRNA regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart an mRNA cleaving activity to the molecule.

In a related aspect, the invention features a mammalian cell which includes an enzymatic RNA molecule as described above. Preferably, the mammalian cell is a human or other primate cell.

In another related aspect, the invention features an expression vector which includes nucleic acid encoding the enzymatic RNA molecules described above, located in the vector, e.g., in a manner which allows expression of that enzymatic RNA molecule within a mammalian cell.

In yet another related aspect, the invention features a method for treatment of an mdr-1 gene-related disease, chronic myelogenous leukemia (CML), promyelocytic leukemia, Burkitt's lymphoma or acute lymphocytic leukemia, follicular lymphoma, B-cell acute lymphocytic leukemia, breast cancer, colon carcinoma, neuroblastoma, or lung cancer, by administering to a patient an enzymatic RNA molecule as described above.

In another related aspect, the invention features a method for treatment of CML by *ex vivo* treatment of blood or marrow cells with an enzymatic RNA molecule as described above.

The invention provides a class of chemical cleaving agents which exhibit a high degree of specificity for the mRNA causative of CML, promyelocytic leukemia, Burkitt's lymphoma or acute lymphocytic leukemia, follicular lymphoma, B-cell acute lymphocytic leukemia, breast cancer, colon carcinoma, and neuroblastoma. If desired, such ribozymes can be designed to target equivalent single-stranded DNAs by methods known in the art. The ribozyme molecule is preferably targeted to a highly conserved sequence region of the mdr-1 mRNA. Such enzymatic RNA molecules can be delivered exogenously to affected cells or endogenously to infected cells. In the preferred hammerhead motif the small size (less than 40 nucleotides, preferably between 32 and 36 nucleotides in length) of the molecule allows the cost of treatment to be reduced compared to other ribozyme motifs.

The smallest ribozyme delivered for any type of treatment reported to date (by Rossi et al., 1992, *supra*) is an *in vitro* transcript having a length of 142 nucleotides. Synthesis of ribozymes greater than 100 nucleotides in length is very difficult using automated methods, and the therapeutic cost of such molecules is prohibitive. Delivery of ribozymes by expression vectors is primarily feasible using only *ex vivo* treatments. This limits the utility of this approach. In this invention,

small ribozyme motifs (e.g., of the hammerhead structure, shown generally in Fig. 1) are used for exogenous delivery. The simple structure of these molecules also increases the ability of the ribozyme to invade targeted regions of the mRNA structure. Thus, unlike the situation when the hammerhead structure is included within longer transcripts, there are no non-ribozyme flanking sequences to interfere with correct folding of the ribozyme structure, as well as complementary binding of the ribozyme to the mRNA target.

The enzymatic RNA molecules of this invention can be used to treat human CML, promyelocytic leukemia, Burkitt's lymphoma, acute lymphocytic leukemia, follicular lymphoma, B-cell acute lymphocytic leukemia, breast cancer, or lung cancer. Affected animals can be treated at the time of cancer, or in a prophylactic manner. This timing of treatment will reduce the number of affected cells and disable cellular replication. This is possible because the ribozymes are designed to disable those structures required for successful cellular proliferation.

Ribozymes of this invention may be used as diagnostic tools to examine genetic drift and mutations within diseased cells. The close relationship between ribozyme activity and the structure of the target RNA allows the detection of mutations in any region of the molecule which alters the base-pairing and three-dimensional structure of the target RNA. By using multiple ribozymes described in this invention, one may map nucleotide changes which are important to RNA structure and function *in vitro*, as well as in cells and tissues. Cleavage of target RNAs with ribozymes may be used to inhibit gene expression and define the role (essentially) of specified gene products in the progression of disease. In this manner, other genetic targets may be defined as important mediators of the disease. These experiments will lead to better treatment of the disease progression by affording the possibility of

combinational therapies (e.g., multiple ribozymes targeted to different genes, ribozymes coupled with known small molecule inhibitors, or intermittent treatment with combinations of ribozymes and/or other chemical or biological molecules).

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The drawings will first briefly be described.

Drawings

Fig. 1 is a diagrammatic representation of a hammerhead motif ribozyme showing stems I, II and III (marked (I), (II) and (III) respectively) interacting with a target region. The 5′ and 3′ ends of both ribozyme and target are shown. Dashes indicate base-paired nucleotides.

Figs. 2 - 11 are preferred targets for mdr-1 gene, chronic myelogenous leukemia, promyelocytic leukemia, Burkitt's lymphoma or acute lymphocytic leukemia, follicular lymphoma, B-cell acute lymphocytic leukemia, breast cancer, colon carcinoma, neuroblastoma, and lung cancer, respectively.

Target Sites

Ribozymes targeting selected regions of mRNA associated with tumor cell growth are preferably chosen to cleave the target RNA in a manner which inhibits translation of the mRNA. Genes are selected such that inhibition of translation will preferably inhibit cell replication, e.g., by inhibiting production of a necessary protein. Selection of effective target sites within these critical regions of mRNA entails testing the accessibility of the target mRNA to hybridization with various oligonucleotide probes. These studies can be performed using RNA or DNA probes and assaying accessibility by cleaving the hybrid molecule with RNaseH (see below). Alternatively, such a study can use ribozyme probes

designed from secondary structure predictions of the mRNAs, and assaying cleavage products by polyacrylamide gel electrophoresis (PAGE), to detect the presence of cleaved and uncleaved molecules.

The following are examples of cancer conditions which can be targetted in this manner.

Chronic Myelogenous Leukemia

Chronic myelogenous leukemia exhibits a characteristic disease course, presenting initially as a chronic granulocytic hyperplasia, and invariably evolving into an acute leukemia which is caused by the clonal expansion of a cell with a less differentiated phenotype (i.e., the blast crisis stage of the disease). CML is an unstable disease which ultimately progresses to a terminal stage which resembles acute leukemia. This lethal disease affects approximately 16,000 patients a year. Chemotherapeutic agents such as hydroxyurea or busulfan can reduce the leukemic burden but do not impact the life expectancy of the patient (e.g., approximately 4 years). Consequently, CML patients are candidates for bone marrow transplantation (BMT) therapy. However, for those patients which survive BMT, disease recurrence remains a major obstacle. Apperley et al., 69 Br. J. Haematol. 239, 1988.

The Philadelphia (Ph) chromosome which results from the translocation of the *abl* oncogene from chromosome 9 to the *bcr* gene on chromosome 22 is found in greater than 95% of CML patients and in 10-25% of all cases of acute lymphoblastic leukemia (ALL). Fourth International Workshop on Chromosomes in Leukemia. 11 Cancer Genet. Cytogenet. 316, 1982. In virtually all Ph-positive CMLs and approximately 50% of the Ph-positive ALLs, the leukemic cells express *bcr-abl* fusion mRNAs in which exon 2 (b2a2 junction) or exon 3 (b3a2 junction) from the major breakpoint cluster region of the *bcr* gene is spliced to exon 2 of the *abl* gene. Heisterkamp et al., 315 Nature 758, 1985, Shtivelman et al., 69 Blood 971, 1987. In the

remaining cases of Ph-positive ALL, the first exon of the *bcr* gene is spliced to exon 2 of the *abl* gene. Hooberman et al., 86 Proc. Natl. Acad. Sci. USA 4259, 1989, Heisterkamp et al., 16 Nucleic Acids Research 10069, 1988.

The b3a2 and b2a2 fusion mRNAs encode 210 kd bcr-abl fusion proteins which exhibit oncogenic activity. Daley et al., 247 Science 824, 1990, Heisterkamp et al., 344 Nature 251, 1990. The importance of the bcr-abl fusion protein ($p210^{bcr-abl}$) in the evolution and maintenance of the leukemic phenotype in human disease has been demonstrated using antisense oligonucleotide inhibition of $p210^{bcr-abl}$ expression. These inhibitory molecules have been shown to inhibit the *in vitro* proliferation of leukemic cells in bone marrow from CML patients. Szczylik et al., 253 Science 562, 1991.

c-Myc Gene

c-Myc, when activated, can induce malignancy in a variety of tissues, most notably hematopoietic tissues (Leder et al., 222 Science 765, 1983). The most common mechanism of c-myc activation is translocation to any of the immunoglobulin (Ig) or T-cell receptor loci during lymphoid maturation (Croce and Nowell, 65 Blood 1, 1985; Klein and Klein, 6 Immunol. Today 208, 1985). For example, in Burkitt's lymphoma the c-myc locus on chromosome 8 translocates most often to the Ig heavy chain locus on chromosome 14, but also to the lambda or kappa light chain Ig genes on chromosomes 2 and 22 (Magrath, in "Epstein-Barr Virus and Associated Diseases", M. Nijhoff Publishing:631, 1986). In some instances the c-myc transcription region is altered in the non-coding exon 1 region; in such cases transcription is initiated at a cryptic promoter present in the first intron of the c-myc locus. These rearrangements are thought to lead to deregulation of c-myc expression.

c-Myc is not normally expressed in quiescent cells, but is temporally expressed in actively-dividing

cells, most prominently during transition from Go to G1 phases of growth induction.

Experiments with transfected cell lines and transgenic animals have shown that c-myc activation plays a critical role, but is not sufficient for transformation (Adams et al., 318 Nature 533, 1985; Lombardi et al., 49 Cell 161, 1987; Schwartz et al., 6 Mol. Cell. Biol. 3221, 1986; Langdon et al., 47 Cell 11, 1986). Targeted inhibition of c-myc expression in tumor cell lines using antisense oligonucleotides has shown that c-myc expression is required for growth in certain lymphomas (McManaway et al., 335 Lancet 808, 1990).

Bcl-2 Gene

The *bcl*-2 gene is abnormally expressed in about 85% of follicular lymphomas and about 20% of diffuse lymphomas due to a t(14;18)(q32;q21) chromosomal rearrangement between the *bcl*-2 locus on chromosome 18 and the immunoglobulin heavy chain locus on chromosome 14 (Yunis et al., 316 N. Engl. J. Med. 79, 1987). This chromosomal rearrangement represents the most common found in lymphoid malignancies in humans. A *bcl*-2/IgH fusion message is expressed; however, the *bcl*-2 protein-coding region is not interrupted since the major breakpoint region lies in the 3' nontranslated region of the *bcl*-2 transcript (Cleary et al., 47 Cell 19, 1986). The *bcl*-2 gene represents a new form of proto-oncogene in that it encodes a mitochondrial protein which inhibits cell senescence (Hockenbery et al., 348 Nature 334, 1990), leading to extended survival of B-cells transfected with this gene (Nunez et al., 86 Proc. Natl. Acad. Sci. USA 4589, 1989).

At least three different forms of *bcl*-2 mRNAs are found in pre-B-cells and T-cells, which vary due to alternative splicing and promoter usage. Two different proteins are produced, a 21 kD and a 26 kD peptide which vary at their carboxytermini. Both forms have identical

N termini encoded in exon 2 of the gene. Consequently, this region would be suitable for ribozyme targeting.

Breast Cancer

The epidermal growth factor (EGF) receptors have been implicated in human cancer more frequently than any other family of growth factor receptors. The EGF receptor gene is often amplified or overexpressed in squamous cell carcinomas and glioblastomas. Jenkins et al., 39 Cancer Genet. Cytogenet. 253, 1989. Similarly, erbB-2 is often overexpressed in adenocarcinomas of the stomach, breast and ovary. Turc-Carel et al., 12 ibid. 1, 1984. Overexpression of either gene under appropriate experimental conditions confers the transformed phenotype. Heim et al., 32 ibid. 13, 1988. In certain breast carcinomas, the erbB-3 gene is overexpressed. Boehm et al., 7 EMBO J. 385, 1988.

The high incidence of human breast cancer has prompted efforts to model the disease in transgenic mice. The myc gene is amplified in some human breast cancers, Escot et al., 83 Proc. Natl. Acad. Sci. USA 4834, 1986, and ras mutations have been observed. Barbacid, 56 Ann. Rev. Biochem. 779, 1987. Reproduction of disease by expression of the myc or ras genes in mice have given only sporadic results.

Breast cancer progression often correlates with amplification of the tyrosine kinase receptor gene denoted as c-erb-B2 or neu. The ligand for this receptor is unknown. Male and female mice expressing the neu gene both synchronously developed adenocarcinomas encompassing the entire gland. Muller et al., 54 Cell 105, 1988. Other strains developed tumors stochastically. Bouchard et al., 57 ibid. 931, 1989.

Colon Carcinoma

The platelet derived growth factor (PDGF) system has served as a prototype for identification of substrates of the receptor tyrosine kinases. Certain enzymes become activated by the PDGF receptor kinase, including

phospholipase C and phosphatidylinositol 3' kinase, Ras guanosine triphosphate (GTPase) activating protein (GAP) and src-like tyrosine kinases. GAP regulates the function of the Ras protein. It stimulates the GTPase activity of the 21 kD Ras protein. Barbacid, 56 Ann. Rev. Biochem. 779, 1987. Microinjection of oncogenically activated Ras into NIH 3T3 cells induces DNA synthesis. Mutations that cause oncogenic activation of ras lead to accumulation of Ras bound to GTP, the active form of the molecule. These mutations block the ability of GAP to convert Ras to the inactive form. Mutations that impair the interactions of Ras with GAP also block the biological function of Ras.

While a number of ras alleles exist (N-ras, K-ras, H-ras) which have been implicated in carcinogenesis, the type most often associated with colon and pancreatic carcinomas is the K-ras. Ribozymes which are targeted to certain regions of the K-ras allelic mRNAs may also prove inhibitory to the function of the other allelic mRNAs of the N-ras and H-ras genes.

Lung Cancer/L-myc Gene

Expression of the myc oncogene is known to alter cell growth in a number of tissues. The product of this gene is a protein which is known to be a transcriptional activator that can act singly or in combination with other oncogene proteins. The L-myc gene is often activated by translocations of DNA from other regions of the genome to the regulatory regions 5' of the myc gene ORF. After transcription of the L-myc mRNA, alternate splicing of the transcript is known to occur. Kaye et al., 8 Mol. Cell Biol. 196, 1988. The alternate mRNAs produced contain a common 5' exon 1 and portions of a common exon 2. These common regions of mRNA structure allow the use of nucleic acid targeted therapeutics which can inactivate both species of mRNA with one therapeutic molecule.

Promyelocytic Leukemia

Acute promyelocytic leukemia is characterized by a specific translocation, a t(15;17)(q22;q11.2-12), which

is found in some 90% of the cases. The t(15;17) is often the only detectable cytogenetic abnormality present in the leukemic cells. This rearrangement results in the fusion of two genes, the promyelocytic leukemia gene (*PML*) on chromosome 15, and the retinoic acid receptor alpha gene (*RARA*) on chromosome 17 (J. Borrow et al., 249 Science 1577, 1990; H. de Thé et al., 347 Nature 558, 1990). The *RARA* is a hormonally-responsive transcriptional regulatory protein, while the function of the *PML* is as yet unknown.

A fusion message is expressed in the leukemic cells which encodes the N-terminal coding region of the *PML* gene and the C-terminal coding region of the *RARA* gene. Expression of this fusion gene apparently inhibits normal myeloid differentiation. The biological relevance of this rearrangement to the etiology of the disease has been exemplified by the discovery that all-trans retinoic acid can be used to achieve complete clinical remission, presumably by inducing differentiation of the leukemic cells. This suggests that the fusion protein is still hormonally responsive.

The treatment of leukemic cells with retinoic acid is not preferable over the long term because retinoic acid is a generalized inducer of differentiation in all cell types, not just leukemic cells. Thus, systemic administration of these compounds can lead to a number of deleterious side effects by differentiating cells which should not be in a differentiated state. A treatment which gives suppression of the transformed phenotype in leukemic cells without affecting other cell types is preferable, as described herein.

B-Cell Acute Lymphocytic Leukemia

Leukemia comprises some 3% of the new cancer cases per year, with lymphocytic leukemias accounting for approximately half (National Cancer Institute, 1990 statistics). A subset of lymphocytic leukemias of the acute pre-B-cell type are associated with a specific chromosomal translocation, a t(1;19)(q23;p13.3) (M.B.

Kamps et al., 60 Cell 547, 1990; J. Nourse et al., *ibid.* p.535). This rearrangement results in the fusion of two genes, the *PBX* gene present on chromosome 1, and the *E2A* gene present on chromosome 19. While the *E2A* transcript is found in all B-cell types, the *PRL* gene is not normally expressed in B-cells. However, an *E2A/PRL* fusion message is constitutively expressed from this aberrant locus in the leukemic cells. This fusion message encodes the N-terminal region of the *E2A*, including the transcriptional activating domain of that gene, and the C-terminal region of the *PRL* gene, which contains a homeodomain DNA binding motif. Thus, a potentially functional chimeric transcriptional regulatory protein is expressed in the leukemic cells.

The *PRL* sequences found in the fusion mRNA are good targets for ribozyme therapy since *PRL* is not expressed in non-leukemic B-cells. Whether the *E2A* sequences can be targeted by ribozymes is unclear since such ribozymes may inhibit *E2A* expression in normal B-cells. It is not known how normal B-cells are affected by inhibition of *E2A*.

The following is but one example of a method by which suitable target sites can be identified and is not limiting in this invention. Generally, the method involves identifying potential cleavage sites for a hammerhead ribozyme, and then testing each of these sites to determine their suitability as targets by ensuring that secondary structure formation is minimal.

The mRNA sequences are compared in an appropriate target region. Putative ribozyme cleavage sites are identified from weak or non-base paired regions of the mRNA. These sites represent the preferred sites for hammerhead or other ribozyme cleavage within these target mRNAs.

Short RNA substrates corresponding to each of the mRNA sites are designed. Each substrate is composed of two to three nucleotides at the 5′ and 3′ ends that

will not base pair with a corresponding ribozyme recognition region. The unpaired regions flank a central region of 12-14 nucleotides to which complementary arms in the ribozyme are designed.

The structure of each substrate sequence is predicted using a PC fold computer program. Sequences which give a positive free energy of binding are accepted. Sequences which give a negative free energy are modified by trimming one or two bases from each of the ends. If the modified sequences are still predicted to have a strong secondary structure, they are rejected.

After substrates are chosen, ribozymes are designed to each of the RNA substrates. Ribozyme folding is also analyzed using PC fold.

Ribozyme molecules are sought which form hammerhead motif stem II (see Fig. 1) regions and contain flanking arms which are devoid of intramolecular base pairing. Often the ribozymes are modified by trimming a base from the ends of the ribozyme, or by introducing additional base pairs in stem II to achieve the desired fold. Ribozymes with incorrect folding are rejected. After substrate/ribozyme pairs are found to contain correct intramolecular structures, the molecules are folded together to predict intermolecular interactions. A schematic representation of a ribozyme with its coordinate base pairing to its cognate target sequence is shown in Fig. 1. Examples of useful targets are listed in Figs. 2 - 11.

Those targets thought to be useful as ribozyme targets can be tested to determine accessibility to nucleic acid probes in a ribonuclease H assay (see below). This assay provides a quick test of the use of the target site without requiring synthesis of a ribozyme. It can be used to screen for sites most suited for ribozyme attack.

Synthesis of Ribozymes

Ribozymes useful in this invention can be produced by gene transcription as described by Cech,

supra, or by chemical synthesis. Chemical synthesis of RNA is similar to that for DNA synthesis. The additional 2'-OH group in RNA, however, requires a different protecting group strategy to deal with selective 3'-5' internucleotide bond formation, and with RNA susceptibility to degradation in the presence of bases. The recently developed method of RNA synthesis utilizing the t-butyldimethylsilyl group for the protection of the 2' hydroxyl is the most reliable method for synthesis of ribozymes. The method reproducibly yields RNA with the correct 3'-5' internucleotide linkages, with average coupling yields in excess of 99%, and requires only a two-step deprotection of the polymer.

A method based upon H-phosphonate chemistry of phosphoramidites gives a relatively lower coupling efficiency than a method based upon phosphoroamidite chemistry. This is a problem for synthesis of DNA as well. A promising approach to scale-up of automatic oligonucleotide synthesis has been described recently for the H-phosphonates. A combination of a proper coupling time and additional capping of "failure" sequences gave high yields in the synthesis of oligodeoxynucleotides in scales in the range of 14 μmoles with as little as 2 equivalents of a monomer in the coupling step. Another alternative approach is to use soluble polymeric supports (e.g., polyethylene glycols), instead of the conventional solid supports. This method can yield short oligonucleotides in hundred milligram quantities per batch utilizing about 3 equivalents of a monomer in a coupling step.

Various modifications to ribozyme structure can be made to enhance the utility of ribozymes. Such modifications will enhance shelf-life, half-life *in vitro*, stability, and ease of introduction of such ribozymes to the target site, e.g., to enhance penetration of cellular membranes, and confer the ability to recognize and bind to targeted cells.

Exogenous delivery of ribozymes benefits from chemical modification of the backbone, e.g., by the overall negative charge of the ribozyme molecule being reduced to facilitate diffusion across the cell membrane. The present strategies for reducing the oligonucleotide charge include: modification of internucleotide linkages by methylphosphonates, use of phosphoramidites, linking oligonucleotides to positively charged molecules, and creating complex packages composed of oligonucleotides, lipids and specific receptors or effectors for targeted cells. Examples of such modifications include sulfur-containing ribozymes containing phosphorothioates and phosphorodithioates as internucleotide linkages in RNA. Synthesis of such sulfur-modified ribozymes is achieved by use of the sulfur-transfer reagent, ^{3}H-1,2-benzenedithiol-3-one 1,1-dioxide. Ribozymes may also contain ribose modified ribonucleotides. Pyrimidine analogues are prepared from uridine using a procedure employing diethylamino sulphur trifluoride (DAST) as a starting reagent. Ribozymes can also be either electrostatically or covalently attached to polymeric cations for the purpose of reducing charge. The polymer can be attached to the ribozyme by simply converting the 3'-end to a ribonucleoside dialdehyde which is obtained by a periodate cleavage of the terminal 2',3'-cis diol system. Depending on the specific requirements for delivery systems, other possible modifications may include different linker arms containing carboxyl, amino or thiol functionalities. Yet further examples include use of methylphosphonates and 2'-O-methylribose and 5' or 3' capping or blocking with m_7GpppG or $m_3^{2,2,7}$GpppG.

For example, a kinased ribozyme is contacted with guanosine triphosphate and guanyltransferase to add a m^3G cap to the ribozyme. After such synthesis, the ribozyme can be gel purified using standard procedure. To ensure that the ribozyme has the desired activity, it may be tested with and without the 5' cap using standard

procedures to assay both its enzymatic activity and its stability.

Synthetic ribozymes, including those containing various modifiers, can be purified by high pressure liquid chromatography (HPLC). Other liquid chromatography techniques, employing reverse phase columns and anion exchangers on silica and polymeric supports may also be used.

There follows an example of the synthesis of one ribozyme. A solid phase phosphoramidite chemistry was employed. Monomers used were 2'-*tert*-butyl-dimethylsilyl cyanoethylphosphoramidities of uridine, N-benzoyl-cytosine, N-phenoxyacetyl adenosine and guanosine (Glen Research, Sterling, VA). Solid phase synthesis was carried out on either an ABI 394 or 380B DNA/RNA synthesizer using the standard protocol provided with each machine. The only exception was that the coupling step was increased from 10 to 12 minutes. The phosphoramidite concentration was 0.1 M. Synthesis was done on a 1 µmole scale using a 1 µmole RNA reaction column (Glen Research). The average coupling efficiencies were between 97% and 98% for the 394 model, and between 97% and 99% for the 380B model, as determined by a calorimetric measurement of the released trityl cation.

Blocked ribozymes were cleaved from the solid support (e.g., CPG), and the bases and diphosphoester moiety deprotected in a sterile vial by dry ethanolic ammonia (2 mL) at 55°C for 16 hours. The reaction mixture was cooled on dry ice. Later, the cold liquid was transferred into a sterile screw cap vial and lyophilized.

To remove the 2'-*tert*-butyl-dimethylsilyl groups from the ribozyme, the residue was suspended in 1 M tetra-n-butylammonium fluoride in dry THF (TBAF), using a 20-fold excess of the reagent for every silyl group, for 16 hours at ambient temperature (about 15-25°C). The reaction was quenched by adding an equal volume of sterile

1 M triethylamine acetate, pH 6.5. The sample was cooled and concentrated on a SpeedVac to half the initial volume.

The ribozymes were purified in two steps by HPLC on a C4 300 Å 5 mm DeltaPak column in an acetonitrile gradient.

The first step, or "trityl on" step, was a separation of 5'-DMT-protected ribozyme(s) from failure sequences lacking a 5'-DMT group. Solvents used for this step were: A (0.1 M triethylammonium acetate, pH 6.8) and B (acetonitrile). The elution profile was: 20% B for 10 minutes, followed by a linear gradient of 20% B to 50% B over 50 minutes, 50% B for 10 minutes, a linear gradient of 50% B to 100% B over 10 minutes, and a linear gradient of 100% B to 0% B over 10 minutes.

The second step was a purification of a completely deblocked ribozyme by a treatment of 2% trifluoroacetic acid on a C4 300 Å 5 mm DeltaPak column in an acetonitrile gradient. Solvents used for this second step were: A (0.1 M triethylammonium acetate, pH 6.8) and B (80% acetonitrile, 0.1 M triethylammonium acetate, pH 6.8). The elution profile was: 5% B for 5 minutes, a linear gradient of 5% B to 15% B over 60 minutes, 15% B for 10 minutes, and a linear gradient of 15% B to 0% B over 10 minutes.

The fraction containing ribozyme was cooled and lyophilized on a SpeedVac. Solid residue was dissolved in a minimum amount of ethanol and sodium perchlorate in acetone. The ribozyme was collected by centrifugation, washed three times with acetone, and lyophilized.

Expression Vector

While synthetic ribozymes are preferred in this invention, those produced by expression vectors can also be used. In designing a suitable ribozyme expression vector the following factors are important to consider. The final ribozyme must be kept as small as possible to minimize unwanted secondary structure within the ribozyme. A promoter (e.g., a T7, human cytomegalovirus immediate

early (iel), human beta actin, or U6 snRNA promoters) should be chosen to be a relatively strong promoter, and expressible both *in vitro* and *in vivo* (e.g., by co-infection with the T7 RNA polymerase gene, the human cytomegalovirus immediate early (iel) or human beta actin promoters). Such a promoter should express the ribozyme at a level suitable to effect production of enough ribozyme to destroy a target RNA, but not at too high a level to prevent other cellular activities from occurring (unless cell death itself is desired).

A hairpin at the 5' end of the ribozyme is useful to ensure that the required transcription initiation sequence (GG or GGG or GGGAG) does not bind to some other part of the ribozyme and thus affect regulation of the transcription process. The 5' hairpin is also useful to protect the ribozyme from 5'-3' exonucleases. A selected hairpin at the 3' end of the ribozyme gene is useful since it acts as a transcription termination signal, and protects the ribozyme from 3'-5' exonuclease activity. One example of a known termination signal is that present on the T7 RNA polymerase system. This signal is about 30 nucleotides in length. Other 3' hairpins of shorter length can be used to provide good termination and RNA stability. Such hairpins can be inserted within the vector sequences to allow standard ribozymes to be placed in an appropriate orientation and expressed with such sequences attached.

Poly(A) tails are also useful to protect the 3' end of the ribozyme. These can be provided by either including a poly(A) signal site in the expression vector (to signal a cell to add the poly(A) tail *in vivo*), or by introducing a poly(A) sequence directly into the expression vector. In the first approach the signal must be located to prevent unwanted secondary structure formation with other parts of the ribozyme. In the second approach, the poly(A) stretch may reduce in size over time when expressed *in vivo*, and thus the vector may need to be

checked over time. Care must be taken in addition of a poly(A) tail which binds poly(A) binding proteins which prevent the ribozyme from acting.

Ribozyme Testing

Once the desired ribozymes are selected, synthesized and purified, they are tested in kinetic and other experiments to determine their utility. An example of such a procedure is provided below.

Preparation of Ribozyme

Crude synthetic ribozyme (typically 350 µg at a time) was purified by separation on a 15% denaturing polyacrylamide gel (0.75 mm thick, 40 cm long) and visualized by UV shadowing. Once excised, gel slices containing full length ribozyme were soaked in 5 ml gel elution buffer (0.5 M NH_4OAc, 1 mM EDTA) overnight with shaking at 4°C. The eluent was desalted over a C-18 matrix (Sep-Pak cartridges, Millipore, Milford, MA) and vacuum dried. The dried RNA was resuspended in 50-100 µl TE (TRIS 10 mM, EDTA 1 mM, pH 7.2). An aliquot of this solution was diluted 100-fold into 1 ml TE, half of which was used to spectrophotometrically quantitate the ribozyme solution. The concentration of this dilute stock was typically 150-800 nM. Purity of the ribozyme was confirmed by the presence of a single band on a denaturing polyacrylamide gel.

A ribozyme may advantageously be synthesized in two or more portions. Each portion of a ribozyme will generally have only limited or no enzymatic activity, and the activity will increase substantially (by at least 5-10 fold) when all portions are ligated (or otherwise juxtaposed) together. A specific example of hammerhead ribozyme synthesis is provided below.

The method involves synthesis of two (or more) shorter "half" ribozymes and ligation of them together using T4 RNA ligase. For example, to make a 34 mer ribozyme, two 17 mers are synthesized, one is phosphorylated, and both are gel purified. These purified

17 mers are then annealed to a DNA splint strand complementary to the two 17 mers. (Such a DNA splint is not always necessary.) This DNA splint has a sequence designed to locate the two 17 mer portions with one end of each adjacent each other. The juxtaposed RNA molecules are then treated with T4 RNA ligase in the presence of ATP. The 34 mer RNA so formed is then HPLC purified.

Preparation of Substrates

Approximately 10-30 pmoles of unpurified substrate was radioactively 5' end-labeled with T4 polynucleotide kinase using 25 pmoles of [γ-^{32}P] ATP. The entire labeling mix was separated on a 20% denaturing polyacrylamide gel and visualized by autoradiography. The full length band was excised and soaked overnight at 4°C in 100 µl of TE (10 mM Tris-HCl pH 7.6, 0.1 mM EDTA).

Kinetic Reactions

For reactions using short substrates (between 8 and 16 bases) a substrate solution was made 1X in assay buffer (75 mM Tris-HCl, pH 7.6; 0.1 mM EDTA, 10 mM $MgCl_2$) such that the concentration of substrate was less than 1 nM. A ribozyme solution (typically 20 nM) was made 1X in assay buffer and four dilutions were made using 1X assay buffer. Fifteen µl of each ribozyme dilution (i.e., 20, 16, 12, 8 and 4 nM) was placed in a separate tube. These tubes and the substrate tube were pre-incubated at 37°C for at least five minutes.

The reaction was started by mixing 15 µl of substrate into each ribozyme tube by rapid pipetting (note that final ribozyme concentrations were 10, 8, 6, 4, 2 nM). Five µl aliquots were removed at 15 or 30 second intervals and quenched with 5 µl stop solution (95% formamide, 20 mM EDTA xylene cyanol, and bromphenol blue dyes). Following the final ribozyme time point, an aliquot of the remaining substrate was removed as a zero ribozyme control.

The samples were separated on either 15% or 20% polyacrylamide gels. Each gel was visualized and

quantitated with an Ambis beta scanner (Ambis Systems, San Diego, CA).

For the most active ribozymes, kinetic analyses were performed in substrate excess to determine K_m and K_{cat} values.

For kinetic reactions with long RNA substrates (greater than 15 bases in length) the substrates were prepared by transcription using T7 RNA polymerase and defined templates containing a T7 promoter, and DNA encoding appropriate nucleotides of the target RNA. The substrate solution was made 1X in assay buffer (75 mM Tris-HCl, pH 7.6; 0.1 mM EDTA; 10 mM $MgCl_2$) and contained 58 nanomolar concentration of the long RNA molecules. The reaction was started by addition of gel purified ribozymes to 1 μM concentration. Aliquots were removed at 20, 40, 60, 80 and 100 minutes, then quenched by the addition of 5 μl stop solution. Cleavage products were separated using denaturing PAGE. The bands were visualized and quantitated with an Ambis beta scanner.

Kinetic Analysis

A simple reaction mechanism for ribozyme-mediated cleavage is:

$$R + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [R:S] \overset{k_2}{\rightleftharpoons} [R:P] \boxed{\rightarrow R + P}$$

where R = ribozyme, S = substrate, and P = products. The boxed step is important only in substrate excess. Because ribozyme concentration is in excess over substrate concentration, the concentration of the ribozyme-substrate complex ([R:S]) is constant over time except during the very brief time when the complex is being initially formed, i.e.,:

$$\frac{d[R:S]}{dt} = 0$$

where t = time, and thus:

$$(R)(S)k_1 = (RS)(k_2 + k_1).$$

The rate of the reaction is the rate of disappearance of substrate with time:

$$\text{Rate} = \frac{-d(S)}{dt} = k_2(RS)$$

Substituting these expressions:

$$(R)(S)k_1 = 1/k_2 \frac{-d(S)}{dt} (k_2 + k_1)$$

or:

$$\frac{-d(S)}{S} = \frac{k_1k_2}{(k_2 + k_1)} (R)\, dt$$

Integrating this expression with respect to time yields:

$$-\ln \frac{S}{S_0} = \frac{k_1k_2}{(k_2 + k_1)} (R)\, t$$

where S_0 = initial substrate. Therefore, a plot of the negative log of fraction substrate uncut versus time (in minutes) yields a straight line with slope:

$$\text{slope} = \frac{k_1k_2}{(k_2 + k_1)} (R) = k_{obs}$$

where k_{obs} = observed rate constant. A plot of slope (k_{obs}) versus ribozyme concentration yields a straight line with a slope which is:

$$\text{slope} = \frac{k_1k_2}{(k_2 + k_1)} \text{ which is } \frac{k_{cat}}{K_m}$$

Using these equations the data obtained from the kinetic experiments provides the necessary information to determine which ribozyme tested is most useful, or active. Such ribozymes can be selected and tested in *in vivo* or *ex vivo* systems.

Liposome Preparation

Lipid molecules are dissolved in a volatile organic solvent ($CHCl_3$, methanol, diethylether, ethanol, etc.). The organic solvent is removed by evaporation. The lipid is hydrated into suspension with 0.1x phosphate buffered saline (PBS), then freeze-thawed 3x using liquid nitrogen and incubation at room temperature. The

suspension is extruded sequentially through a 0.4 μm, 0.2 μm and 0.1 μm polycarbonate filters at maximum pressure of 800 psi. The ribozyme is mixed with the extruded liposome suspension and lyophilized to dryness. The lipid/ribozyme powder is rehydrated with water to one-tenth the original volume. The suspension is diluted to the minimum volume required for extrusion (0.4 ml for 1.5 ml barrel and 1.5 ml for 10 ml barrel) with 1xPBS and re-extruded through 0.4 μm, 0.2 μm, 0.1 μm polycarbonate filters. The liposome entrapped ribozyme is separated from untrapped ribozyme by gel filtration chromatography (SEPHAROSE CL-4B, BIOGEL A5M). The liposome extractions are pooled and sterilized by filtration through a 0.2 μm filter. The free ribozyme is pooled and recovered by ethanol precipitation. The liposome concentration is determined by incorporation of a radioactive lipid. The ribozyme concentration is determined by labeling with ^{32}P. Rossi et al., 1992, *supra* (and references cited therein) describe other methods suitable for preparation of liposomes.

Examples of other useful liposome preparations which display similar degrees of uptake of both a radioactive lipid marker and an entrapped fluorophore by Vero cells showed different fluorescent staining patterns. Specifically, liposomes composed of DPPG/DPPC/Cholesterol (in a ratio of: 50/17/33) gave a punctate pattern of fluorescence, while DOPE/Egg PC/Cholesterol (30/37/33) gave a diffuse, homogeneous pattern of fluorescence in the cytoplasm. Cell fractionation showed that 80% of the entrapped contents from the DPPG/DPPC/Cholesterol formulation was localized in the membrane fraction, whereas the DOPE/Egg PC/Cholesterol formulation was localized in the cytoplasm. Further characterization of the latter formulation showed that after 3 hours, 70% of the fluorescence was cytoplasmic and 30% was in the membrane. After 24 hours, uptake had increased 5-fold and

the liposome contents were distributed 50/50 between the cytoplasmic and membrane fractions.

Liposomes containing 15 ribozymes (^{32}P-labeled) targeted to the HSV ICP4 mRNA were prepared and incubated with the cells. After 24 hours, 25% of the liposome dose was taken up with approximately 60,000 liposomes per cell. Thirty percent of the delivered ribozyme was intact after 24 hours. Cell fractionation studies showed 40% of the intact ribozyme to be in the membrane fraction and 52% of the intact ribozyme to be in the cytoplasmic fraction.

In Vivo Assay

The efficacy of action of a chosen ribozyme may be tested *in vivo* by use of cell cultures sensitive to mdr-1 gene expression, using standard procedures in transformed cells or animals which express the target mRNA using standard procedures.

The efficacy of action of a chosen ribozyme may be tested in tissue culture by use of transformed cells containing the target mRNA (e.g., K562 cells which express the b3a2 fusion mRNA) using standard procedures. Alternatively, ribozyme efficacy could be tested with peripheral blood or bone marrow from CML patients using soft-agar colony forming assays. Such methods are known to those educated in this field.

Ribonuclease Protection Assay

The accumulation of target mRNA in cells or the cleavage of the mRNA by ribozymes or RNaseH (*in vitro* or *in vivo*) can be quantified using an RNase protection assay.

In this method, antisense riboprobes are transcribed from template DNA using T7 RNA polymerase (U.S. Biochemical) in 20 µl reactions containing 1X transcription buffer (supplied by the manufacturer), 0.2 mM ATP, GTP and UTP, 1 U/µl pancreatic RNase inhibitor (Boehringer Mannheim Biochemicals) and 200 µCi ^{32}P-labeled CTP (800 Ci/mmol, New England Nuclear) for 1 hour at 37°C. Template DNA is digested with 1 U RNase-free DNaseI (U.S.

Biochemical, Cleveland, OH) at 37°C for 15 minutes and unincorporated nucleotides removed by G-50 SEPHADEX spin chromatography.

In a manner similar to the transcription of antisense probe, the target mRNA can be transcribed *in vitro* using a suitable DNA template. The transcript is purified by standard methods and digested with ribozyme at 37°C according to methods described later.

Alternatively, afflicted (mRNA-expressing) cells expressing the target mRNA *bcr-abl* fusion transcript are harvested into 1 ml of PBS, transferred to a 1.5 ml EPPENDORF tube, pelleted for 30 seconds at low speed in a microcentrifuge, and lysed in 70 µl of hybridization buffer (4 M guanidine isothiocyanate, 0.1% sarcosyl, 25 mM sodium citrate, pH 7.5). Cell lysate (45 µl) or defined amounts of *in vitro* transcript (also in hybridization buffer) is then combined with 5 µl of hybridization buffer containing 5 x 10^5 cpm of each antisense riboprobe in 0.5 ml EPPENDORF tubes, overlaid with 25 µl mineral oil, and hybridization accomplished by heating overnight at 55°C. The hybridization reactions are diluted into 0.5 ml RNase solution (20 U/ml RNaseA, 2 U/ml RNaseT1, 10 U/ml RNase-free DNaseI in 0.4 M NaCl), heated for 30 minutes at 37°C, and 10 µl of 20% SDS and 10 µl of Proteinase K (10 mg/ml) added, followed by an additional 30 minutes incubation at 37°C. Hybrids are partially purified by extraction with 0.5 ml of a 1:1 mixture of phenol/chloroform; aqueous phases are combined with 0.5 ml isopropanol, and RNase-resistant hybrids pelleted for 10 minutes at room temperature (about 20°C) in a microcentrifuge. Pellets are dissolved in 10 µl loading buffer (95% formamide, 1X TBE, 0.1% bromophenol blue, 0.1% xylene cylanol), heated to 95°C for five minutes, cooled on ice, and analyzed on 4% polyacrylamide/7 M urea gels under denaturing conditions.

Ribozyme Stability

The chosen ribozyme can be tested to determine its stability, and thus its potential utility. Such a test can also be used to determine the effect of various chemical modifications (e.g., addition of a poly(A) tail) on the ribozyme stability and thus aid selection of a more stable ribozyme. For example, a reaction mixture contains 1 to 5 pmoles of 5' (kinased) and/or 3' labeled ribozyme, 15 µg of cytosolic extract and 2.5 mM $MgCl_2$ in a total volume of 100 µl. The reaction is incubated at 37°C. Eight µl aliquots are taken at timed intervals and mixed with 8 µl of a stop mix (20 mM EDTA, 95% formamide). Samples are separated on a 15% acrylamide sequencing gel, exposed to film, and scanned with an Ambis.

A 3'-labeled ribozyme can be formed by incorporation of the ^{32}P-labeled cordycepin at the 3' OH using poly(A) polymerase. For example, the poly(A) polymerase reaction contains 40 mM Tris, pH 8, 10 mM $MgCl_2$, 250 mM NaCl, 2.5 mM $MnCl_2$; 3 µl ^{32}P cordycepin, 500 Ci/mM; and 6 units poly(A) polymerase in a total volume of 50 µl. The reaction mixture is incubated for 30 minutes at 37°C.

Effect of Base Substitution upon Ribozyme Activity

To determine which primary structural characteristics could change ribozyme cleavage of substrate, minor base changes can be made in the substrate cleavage region recognized by a specific ribozyme. For example, the substrate sequences can be changed at the central "C" nucleotide, changing the cleavage site from a GUC to a GUA motif. The K_{cat}/K_m values for cleavage using each substrate are then analyzed to determine if such a change increases ribozyme cleavage rates. Similar experiments can be performed to address the effects of changing bases complementary to the ribozyme binding arms. Changes predicted to maintain strong binding to the complementary substrate are preferred. Minor changes in nucleotide content can alter ribozyme/substrate

interactions in ways which are unpredictable based upon binding strength alone. Structures in the catalytic core region of the ribozyme recognize trivial changes in either substrate structure or the three dimensional structure of the ribozyme/substrate complex.

To begin optimizing ribozyme design, the cleavage rates of ribozymes containing varied arm lengths, but targeted to the same length of short RNA substrate can be tested. Minimal arm lengths are required and effective cleavage varies with ribozyme/substrate combinations.

The cleavage activity of selected ribozymes can be assessed using target mRNA-homologous substrates. The assays are performed in ribozyme excess and approximate K_{cat}/K_{min} values obtained. Comparison of values obtained with short and long substrates indicates utility *in vivo* of a ribozyme.

Intracellular Stability of Liposome-Delivered Ribozymes

To test the stability of a chosen ribozyme *in vivo* the following test is useful. Ribozymes are ^{32}P-end-labeled, entrapped in liposomes and delivered to target mRNA containing cells for three hours. The cells are fractionated and ribozyme is purified by phenol/chloroform extraction. Alternatively, cells ($1x10^7$, T-175 flask) are scraped from the surface of the flask are cultured and washed twice with cold PBS. The cells are homogenized by douncing 35 times in 4 ml of TSE (10 mM Tris, pH 7.4, 0.25 M Sucrose, mM EDTA). Nuclei are pelleted at 100xg for 10 minutes. Subcellular organelles (the membrane fraction) are pelleted at 200,000xg for two hours using an SW60 rotor. The pellet is resuspended in 1 ml of H buffer (0.25 M Sucrose, 50 mM HEPES, pH 7.4). The supernatant contains the cytoplasmic fraction (in approximately 3.7 ml). The nuclear pellet is resuspended in 1 ml of 65% sucrose in TM (50 mM Tris, pH 7.4, 2.5 mM $MgCl_2$) and banded on a sucrose step gradient (1 ml nuclei in 65% sucrose TM, 1 ml 60% sucrose TM, 1 ml 55% sucrose

TM, 50% sucrose TM, 300 µl 25% sucrose TM) for one hour at 37,000xg with an SW60 rotor. The nuclear band is harvested and diluted to 10% sucrose with TM buffer. Nuclei are pelleted at 37,000xg using an SW60 rotor for 15 minutes and the pellet resuspended in 1 ml of TM buffer. Aliquots are size fractionated on denaturing polyacrylamide gels and the intracellular localization determined. By comparison to the migration rate of newly synthesized ribozyme, the various fractions containing intact ribozyme can be determined.

To investigate modifications which would lengthen the half-life of ribozyme molecules intracellularly, the cells may be fractioned as above and the purity of each fraction assessed by assaying enzyme activity known to exist in that fraction.

The various cell fractions are frozen at -70°C and used to determine relative nuclease resistances of modified ribozyme molecules. Ribozyme molecules may be synthesized with 5 phosphorothioate (ps), or 2'-O-methyl (2'-OMe) modifications at each end of the molecule. These molecules and a phosphodiester version of the ribozyme are end-labeled with ^{32}P and ATP using T4 polynucleotide kinase. Equal concentrations are added to the cell cytoplasmic extracts and aliquots of each taken at 10 minute intervals. The samples are size fractionated by denaturing PAGE and relative rates of nuclease resistance analyzed by scanning the gel with an Ambis β-scanner. The results show whether the ribozymes are digested by the cytoplasmic extract, and which versions are relatively more nuclease resistant. Modified ribozymes generally maintain 80-90% of the catalytic activity of the native ribozyme when short RNA substrates are employed.

Unlabeled, 5' end-labeled or 3' end-labeled ribozymes can be used in the assays. These experiments can also be performed with human cell extracts to verify the observations.

In one example, Vero or HeLa cells were grown to 90-95% confluency in 175 cm^2 tissue culture flasks, scraped into 10 ml of cold phosphate buffered saline (PBS), then washed once in 10 ml of cold PBS and once in 10 ml of cold TSE (10 mM Tris, pH 7.4; 0.25 M sucrose; 1 mM EDTA). The cell pellets were resuspended in 4 ml of TSE, dounced 35x on ice, and the released nuclei pelleted by centrifugation at 1000g for 10 minutes. The nuclear pellet was resuspended in 1 ml of 65% sucrose TM (50 mM Tris, pH 7.4; 2.5 mM $MgCl_2$) and transferred to Beckman ultra-clear tubes. The following sucrose TM solutions were layered on top of the sample: 1 ml 60%, 1 ml 55%, and 25% sucrose to the top of the tube. Gradients were spun in an SW60 rotor at 37,000g for 1 hour. HeLa nuclei banded at the 55-60% sucrose boundary and Vero nuclei banded at the 50-55% sucrose boundary. Nuclear bands were harvested, diluted to 10% sucrose with TM buffer, and pelleted by centrifugation at 37,000g for 15 minutes using an SW60 rotor. The nuclear pellet was resuspended in 1 ml of TM buffer. Subcellular organelles and membrane components in the post nuclear supernatant were separated from the cytoplasmic fraction by centrifugation at 200,000g for 2 hours in an SW60 rotor. The pellet contained the membrane fraction, which was resuspended in 1 ml of H buffer (0.25 M sucrose; 50 mM HEPES, pH 7.4), and the supernatant contained the cytoplasmic fraction.

Purity of the various fractions was assessed using enzymatic markers specific for the cytoplasmic and membranous fractions. Three enzyme markers for the membranous fraction were used; hexosaminidase and β-glucocerebrosidase are localized in lysosomes, while alkaline phosphodiesterase is specific to endosomes. Specifically, the assays were as follows:

For N-acetyl-beta-hexosaminidase, the reaction mixture contained 0.3 mg/ml 4-methylumbelliferyl-N-acetyl-glucosaminide; 20 mM sodium citrate; pH 4.5; 0.01% Triton X-100; and 100 µl of sample in a final volume of 500 µl

(Harding et al., 64 Cell 393, 1991). The reactions were incubated at 37°C for 1 hour and stopped by the addition of 1.5 ml of stop buffer (0.13 M glycine, 0.07 M NaCl, 0.08 M sodium carbonate, pH 10.6). The reaction product was quantitated in a Hitachi F-4010 fluorescence spectrophotometer by excitation of the fluorophore at 360 nm and analysis of the emission at 448 nm.

For Alkaline Phosphodiesterase, the assay medium contained 25 mM CAPS (3-(Cyclohexylamino)-propanesulfonic acid), pH 10.6; 0.05% Triton X-100; 15 mM $MgCl_2$; 1.25 mg/ml Thymidine-5'-monophosphate-p-nitrophenyl ester; and 100 µl of sample in a total reaction volume of 200 µl. The reactions were incubated at 37°C for 2 hours, then diluted to 1 ml with H_2O and the absorbance was measured at 400 nm (Razell and Khorana, 234 J. Biol. Chem. 739, 1959).

For β-glucocerebrosidase, the reaction contained 85 nM sodium citrate, pH 5.9; 0.12% Triton X-100; 0.1% sodium taurocholate; 5 mM 4-methylumbelliferyl β-D-glucopyranoside; and 125 µl of sample in a total volume of 250 µl (Kennedy and Cooper, 252 Biochem. J. 739, 1988). The reaction was incubated at 37°C for 1 hour and stopped by the addition of 0.75 ml of stop buffer. Product formation was measured in a fluorescence spectrophotometer by using an excitation wavelength of 360 nm and analysis of the emission at 448 nm.

The cytoplasmic enzyme marker, lactate dehydrogenase, was assayed in an assay mixture containing 0.2 M Tris; pH 7.4; 0.22 mM NADH; 1 mM sodium pyruvate; and 50 µl of sample in a final volume of 1.05 ml. Enzyme levels were determined by decreased absorbency at 350 nm resulting from the oxidation of NADH at room temperature (Silverstein and Boyer, 239 J. Biol. Chem. 3901, 1964).

Lactate dehydrogenase was found predominantly in the cytoplasmic fractions of both Vero and HeLa cells, while β-glucocerebrosidase and alkaline phosphodiesterase were found almost exclusively in the membranous fractions. The hexosaminidase activity in Vero cell fractions was

concentrated in the membranous fraction (70%) with about 20% in the cytoplasmic fraction. The isolation of enzyme markers with the appropriate cellular compartment demonstrated that cytoplasmic, membranous and nuclear fractions can be isolated with minimal intercompartmental contamination using this fractionation scheme.

Nuclease Stability of Ribozymes and mRNA

The simplest and most sensitive way to monitor nuclease activity in cell fractions is to use end-labeled oligonucleotides. However, high levels of phosphatase activity in some biological extracts gives ambiguous results in nuclease experiments when ^{32}P-5'-end-labeled oligonucleotides are used as substrates. To determine the phosphatase activity in the extracts, cellular fractions were incubated with cold ribozymes and trace amounts of 5'-end-labeled ribozyme in the presence of 1 mM Mg^{+2} (or Zn^{+2} with HeLa cytoplasmic extracts) to optimize digestion. After polyacrylamide gel electrophoresis of samples, digestion of the oligonucleotide was assessed both by staining and by autoradiography.

Specifically, the basic oligonucleotide digestion reaction contained substrate nucleic acid (an RNA oligonucleotide of 36 nucleotides) and cell fraction extract in a total volume of 100 µl. Aliquots (7 µl) were taken after various periods of incubation at 37°C and added to 7 µl of gel loading buffer (95% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, and 20 mM EDTA). The samples were separated by electrophoresis on a 7 M urea, 20% polyacrylamide gel. Intact ribozymes were visualized either by staining with Stains-all (United States Biochemical, Cleveland, OH), or autoradiography of ^{32}P-labeled ribozyme. The stained gels and X-ray films were scanned on a Bio 5000 density scanner (U.S. Biochemical). Ribozymes were 5' end-labeled with T4 polynucleotide kinase (U.S. Biochemical) using 10 µCi of ^{32}P γ-ATP (3,000 Ci/mmole, New England Nuclear, Boston, MA), and 20-25 pmoles of ribozymes. The unincorporated

nucleotides were separated from the product by G-50 spin chromatography. Nuclease assays contained 1-2 pmoles of ^{32}P-labeled ribozyme. All oligonucleotides were synthesized on an Applied Biosystems 394 DNA/RNA synthesizer (Applied Biosystems Inc., Foster City, CA) according to manufacturer's protocols. The nuclear fractions were resuspended in a buffer containing 2.5 mM $MgCl_2$. Experiments involving the nuclear fractions were performed in the presence of 1 mM Mg^{+2}, or in combination with 1 mM Mn^{+2}, Ca^{+2}, or Zn^{+2}.

To measure the stability of mRNA, Vero cells were infected with herpes simplex virus (HSV) at a M.O.I. of 5 and total RNA was extracted (Chomczynski and Sacchi, 162 Anal. Biochem. 156, 1987). An RNase protection assay was used to detect mRNA after incubation of total infected cellular RNA in cytoplasmic extracts. RNA probes were produced from PCR-amplified template DNA using T7 RNA polymerase (U.S. Biochemical) in the presence of ^{32}P α-CTP (3,000 Ci/mmole, New England Nuclear, Boston, MA). Template DNA was inactivated with 1 unit of RNase-free DNaseI for 15 minutes at 37°C. Unincorporated nucleotides were removed by G-50 spin chromatography. Samples (6 µl) were taken from the nuclease assays after various periods of incubation at 37°C, added to 40 µl of 4 M GUSCN buffer (4 M guanidinium thiocyanate; 25 mM sodium citrate, pH 7; 0.5% sarcosyl; and 0.1 M 2-mercaptoethanol), and 5 µl of ^{32}P-labeled RNA probe ($5x10^5$ cpm/5 µl, specific activity of $1.8x10^6$ cpm/µg) in 4 M GUSCN buffer. Hybridization reactions were covered with mineral oil and incubated at 55°C for 12-16 hours, after which the hybridization reaction was mixed with 500 µl of RNase buffer (0.4 M NaCl, 20 µg/ml RNaseA, 2 units/ml T1 RNase) and incubated for 30 minutes at 37°C. RNase activity was quenched by incubation with 10 µl of 20% SDS and 10 µl of proteinase K (20 mg/ml), and the RNA was extracted using a phenol/chloroform mixture. The protected RNA fragment was purified by precipitation with an equal volume of

isopropanol in the presence of 20 µg of carrier yeast tRNA. The RNA pellets were resuspended in gel loading buffer, heated to 95°C for 5 minutes and separated by electrophoresis on a 5% polyacrylamide, 7 M urea gel. Protected fragments were visualized by autoradiography, and the films were scanned with a Bio 5000 density scanner.

In experiments using these methods, the rate of digestion of ribozymes in Vero cell extracts was similar, demonstrating the lack of significant phosphatase activity in Vero cellular fractions. Similar results were observed with HeLa cellular fractions. In most extracts, ladders of digested fragments were observed; such ladders would not be expected if digestion was an artifact of phosphatase action. Thus, digestion using 5' end-labeled ribozymes is an accurate assessment of nuclease action in cellular extracts.

In other experiments, labeled ribozymes were incubated in various Vero and HeLa cellular fractions. Incubation of ribozymes in either membranous or nuclear fractions resulted in a linear decrease of intact molecules over time. In contrast, no digestion of ribozymes occurred during a 24 hour incubation in Vero cytoplasmic extracts, and HeLa cytoplasmic extracts exhibited a 20-30 minute delay in the onset of RNA digestion. After this refractory period, the rate of digestion was linear but not as rapid as the rates observed in any of the nuclear or membranous fractions.

The effect of four divalent cations (Mg^{+2}, Mn^{+2}, Ca^{+2}, and Zn^{+2}) on the nuclease activity of the cellular fractions was assessed. Vero cytoplasmic extracts were stimulated by the addition of 1 mM Mg^{+2} or Mn^{+2}, while Ca^{+2} or Zn^{+2} had no effect. Nuclease activity in HeLa cytoplasmic extracts was enhanced only by the addition of 1 mM Zn^{+2}. Both Vero and HeLa membranous fractions exhibited maximum nuclease activity with the addition of Mg^{+2} or Mn^{+2} ions, while the addition of Ca^{+2} significantly

reduced activity of the HeLa membranous fraction and abolished nuclease activity in the Vero membranous fraction. Addition of Zn^{+2} to both membranous fractions resulted in a loss of all RNase activity. The Vero nuclear extract demonstrated roughly equivalent nuclease activity in the presence of either Mg^{+2} alone or a Mg^{+2} and Mn^{+2} ion combination, less in the presence of Mg^{+2} and Ca^{+2}, and no activity in the presence of Mg^{+2} and Zn^{+2}. The effects of cation addition were not as dramatic with HeLa nuclear extracts. The nuclease activity of these fractions was greatest in the presence of Mg^{+2} alone or Mg^{+2} and Ca^{+2} and decreased slightly with the addition of Mn^{+2} or Zn^{+2} to the Mg^{+2} present in the extracts.

To verify that nuclease activity was dependent upon added divalent cations, nuclease assays were performed using 1 mM Mg^{+2} in the presence and absence of 20 mM EDTA. For the HeLa cytoplasmic fractions, the Mg^{+2} was replaced with 1 mM Zn^{+2}. The presence of 20 mM EDTA completely abolished nuclease activity in the Vero and HeLa cytoplasmic fractions and Vero nuclear fractions. Nuclease activity in the HeLa membranous and nuclear fractions was partially inhibited by the addition of EDTA, while EDTA had no effect on the nuclease activity in the Vero membranous fraction. For comparative purposes, reactions using DNA oligonucleotides were performed using different Vero fractions. All DNase activity in Vero cytoplasmic, membranous, and nuclear fractions was inhibited by 20 mM EDTA.

The stability of RNA oligonucleotides and HSV-1 mRNA were compared in the presence and absence of activity-enhancing divalent cations (1 mM Mg^{+2}, Vero cells; 1 mM Zn^{+2}, HeLa cells). Total cellular RNA from HSV-1 infected Vero cells (8 mg) and tracer amounts of ^{32}P-5'-end-labeled RNA oligonucleotides (1 pmole) were incubated with Vero or HeLa cytoplasmic extracts. In the absence of divalent cations, no substantial decrease of intact ribozymes was detected in assays, although mRNA was

digested in both Vero and HeLa cytoplasmic extracts. After addition of divalent cations, digestion of ribozymes occurred in both Vero and HeLa cytoplasmic fractions. The rate of ribozyme digestion in HeLa extracts increased to levels similar to those observed with mRNA, while the rate of mRNA digestion remained greater than the rate of ribozyme digestion in Vero cytoplasmic fractions.

Thus, the stability of hammerhead ribozymes were compared in both Vero and HeLa cell cytoplasmic, membranous and nuclear fractions. Vero cytoplasmic and nuclear fractions were found to require Mg^{+2} for optimal nuclease activity, while the membranous fraction was not altered by the addition of divalent cations. HeLa membranous and nuclear fractions were also activated by Mg^{+2}, while the cytoplasmic fractions required Zn^{+2} for nuclease activation. Relative stabilities of ribozymes and mRNAs were compared in Vero and HeLa cytoplasmic fractions. In the absence of appropriate divalent cations, little ribozyme digestion was observed in either cytoplasmic preparation while mRNA was rapidly digested. The addition of Mg^{+2} to Vero cytoplasmic extracts and Zn^{+2} to the HeLa cytoplasmic extracts stimulated ribozyme degradation and enhanced mRNA digestion. These data show that the nuclease sensitivity of ribozymes is cell-type specific, varies with the intracellular compartment studied and may not be able to be predicted from studies with mRNA. Notably, however, ribozymes appear stable in such cellular fractions for a period of time potentially sufficient to have a therapeutically useful activity.

Administration of Ribozyme

Selected ribozymes can be administered prophylactically, or to patients expressing mdr-1 mRNA, or having CML, leukemic conditions, Burkitt's lymphoma, follicular lymphoma, breast cancer, colon carcinoma, neuroblastoma, lung cancer, or pretumor cells, e.g., by exogenous delivery of the ribozyme to an a desired tissue by means of an appropriate delivery vehicle, e.g., a

liposome, a controlled release vehicle, by use of iontophoresis, electroporation or ion paired molecules, or covalently attached adducts, and other pharmacologically approved methods of delivery. Routes of administration include intramuscular, aerosol, oral (tablet or pill form), topical, systemic, ocular, intraperitoneal and/or intrathecal. Alternatively, ribozymes may be administered to a tissue or afflicted cell *ex vivo* to eradicate tumorigenic cells prior to re-implantation (e.g., in the course of autologous bone marrow transplantation therapy). Expression vectors for immunization with ribozymes and/or delivery of ribozymes are also suitable.

The specific delivery route of any selected ribozyme will depend on the use of the ribozyme. Generally, a specific delivery program for each ribozyme will focus on unmodified ribozyme uptake with regard to intracellular localization, followed by demonstration of efficacy. Alternatively, delivery to these same cells in an organ or tissue of an animal can be pursued. Uptake studies will include uptake assays to evaluate cellular ribozyme uptake, regardless of the delivery vehicle or strategy. Such assays will also determine the intracellular localization of the ribozyme following uptake, ultimately establishing the requirements for maintenance of steady-state concentrations within the cellular compartment containing the target sequence (nucleus and/or cytoplasm). Efficacy and cytotoxicity can then be tested. Toxicity will not only include cell viability but also cell function.

Some methods of delivery that may be used include:

a. encapsulation in liposomes,
b. transduction by retroviral vectors,
c. conjugation with cholesterol,
d. localization to nuclear compartment utilizing nuclear targeting site found on most nuclear proteins,

e. neutralization of charge of ribozyme by using nucleotide derivatives,

f. use of blood stem cells to distribute ribozymes throughout the body, and

g. electroporation.

At least three types of delivery strategies are useful in the present invention, including: ribozyme modifications, particle carrier drug delivery vehicles, and retroviral expression vectors. Unmodified ribozymes, like most small molecules, are taken up by cells, albeit slowly. To enhance cellular uptake, the ribozyme may be modified essentially at random, in ways which reduce its charge but maintains specific functional groups. This results in a molecule which is able to diffuse across the cell membrane, thus removing the permeability barrier.

Modification of ribozymes to reduce charge is just one approach to enhance the cellular uptake of these larger molecules. The random approach, however, is not advisable since ribozymes are structurally and functionally more complex than small drug molecules. The structural requirements necessary to maintain ribozyme catalytic activity are well understood by those in the art. These requirements are taken into consideration when designing modifications to enhance cellular delivery. The modifications are also designed to reduce susceptibility to nuclease degradation. Both of these characteristics should greatly improve the efficacy of the ribozyme. Cellular uptake can be increased by several orders of magnitude without having to alter the phosphodiester linkages necessary for ribozyme cleavage activity.

Chemical modifications of the phosphate backbone will reduce the negative charge allowing free diffusion across the membrane. This principle has been successfully demonstrated for antisense DNA technology. The similarities in chemical composition between DNA and RNA make this a feasible approach. In the body, maintenance of an external concentration will be necessary to drive

the diffusion of the modified ribozyme into the cells of the tissue. Administration routes which allow the diseased tissue to be exposed to a transient high concentration of the drug, which is slowly dissipated by systemic adsorption are preferred. Intravenous administration with a drug carrier designed to increase the circulation half-life of the ribozyme can be used. The size and composition of the drug carrier restricts rapid clearance from the blood stream. The carrier, made to accumulate at the site of infection, can protect the ribozyme from degradative processes.

Drug delivery vehicles are effective for both systemic and topical administration. They can be designed to serve as a slow release reservoir, or to deliver their contents directly to the target cell. An advantage of using direct delivery drug vehicles is that multiple molecules are delivered per uptake. Such vehicles have been shown to increase the circulation half-life of drugs which would otherwise be rapidly cleared from the blood stream. Some examples of such specialized drug delivery vehicles which fall into this category are liposomes, hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres.

From this category of delivery systems, liposomes are preferred. Liposomes increase intracellular stability, increase uptake efficiency and improve biological activity.

Liposomes are hollow spherical vesicles composed of lipids arranged in a similar fashion as those lipids which make up the cell membrane. They have an internal aqueous space for entrapping water soluble compounds and range in size from 0.05 to several microns in diameter. Several studies have shown that liposomes can deliver RNA to cells and that the RNA remains biologically active.

For example, a liposome delivery vehicle originally designed as a research tool, Lipofectin, has

been shown to deliver intact mRNA molecules to cells yielding production of the corresponding protein.

Liposomes offer several advantages: They are non-toxic and biodegradable in composition; they display long circulation half-lives; and recognition molecules can be readily attached to their surface for targeting to tissues. Finally, cost effective manufacture of liposome-based pharmaceuticals, either in a liquid suspension or lyophilized product, has demonstrated the viability of this technology as an acceptable drug delivery system.

Other controlled release drug delivery systems, such as nonoparticles and hydrogels may be potential delivery vehicles for a ribozyme. These carriers have been developed for chemotherapeutic agents and protein-based pharmaceuticals, and consequently, can be adapted for ribozyme delivery.

Topical administration of ribozymes is advantageous since it allows localized concentration at the site of administration with minimal systemic adsorption. This simplifies the delivery strategy of the ribozyme to the disease site and reduces the extent of toxicological characterization. Furthermore, the amount of material to be applied is far less than that required for other administration routes. Effective delivery requires the ribozyme to diffuse into the infected cells or through the skin to the underlying vasculature. Chemical modification of the ribozyme to neutralize negative charge may be all that is required for penetration. However, in the event that charge neutralization is insufficient, the modified ribozyme can be co-formulated with permeability enhancers, such as Azone or oleic acid, in a liposome. The liposomes can either represent a slow release presentation vehicle in which the modified ribozyme and permeability enhancer transfer from the liposome into the infected cell, or the liposome phospholipids can participate directly with the modified ribozyme and permeability enhancer in

facilitating cellular delivery. In some cases, both the ribozyme and permeability enhancer can be formulated into a suppository formulation for slow release.

Ribozymes may also be systemically administered. Systemic absorption refers to the accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include: intravenous, subcutaneous, intraperitoneal, intranasal, intrathecal and ophthalmic. Each of these administration routes expose the ribozyme to an accessible diseased tissue. Subcutaneous administration drains into a localized lymph node which proceeds through the lymphatic network into the circulation. The rate of entry into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier localizes the ribozyme at the lymph node. The ribozyme can be modified to diffuse into the cell, or the liposome can directly participate in the delivery of either the unmodified or modified ribozyme to the cell.

A liposome formulation containing phosphatidyl-ethanolomidomethylthiosuccinimide which can deliver oligonucleotides to lymphocytes and macrophages is also useful for certain cancerous conditions. Furthermore, a 200 nm diameter liposome of this composition was internalized as well as 100 nm diameter liposomes. The 200 nm liposomes exhibit a 10-fold greater packaging capacity than the 100 nm liposomes and can accomodate larger molecules such as a ribozyme expression vector. This oligonucleotide delivery system inhibits viral proliferation in these viruses that infect primary immune cells. This oligonucleotide delivery system prevents mRNA expression in affected primary immune cells. Whole blood studies show that the formulation is taken up by 90% of the lymphocytes after 8 hours at 37°C. Preliminary biodistribution and pharmacokinetic studies yielded 70% of

the injected dose/gm of tissue in the spleen after 1 hour following intravenous administration.

Intraperitoneal administration also leads to entry into the circulation with the molecular weight or size controlling the rate of entry.

Liposomes injected intravenously show accumulation in the liver, lung and spleen. The composition and size can be adjusted so that this accumulation represents 30% to 40% of the injected dose. The remaining dose circulates in the blood stream for up to 24 hours.

The chosen method of delivery should result in cytoplasmic accumulation and molecules should have some nuclease-resistance for optimal dosing. Nuclear delivery may be used but is less preferable. Most preferred delivery methods include liposomes (10-400 nm), hydrogels, controlled-release polymers, microinjection or electroporation (for *ex vivo* treatments) and other pharmaceutically applicable vehicles. The dosage will depend upon the disease indication and the route of administration but should be between 100-200 mg/kg of body weight/day. The duration of treatment will extend through the course of the disease symptoms, possibly continuously. The number of doses will depend upon disease delivery vehicle and efficacy data from clinical trials.

Establishment of therapeutic levels of ribozyme within the cell is dependent upon the rate of uptake and degradation. Decreasing the degree of degradation will prolong the intracellular half-life of the ribozyme. Thus, chemically modified ribozymes, e.g., with modification of the phosphate backbone, or capping of the 5′ and 3′ ends of the ribozyme with nucleotide analogs may require different dosaging. Descriptions of useful systems are provided in the art cited above, all of which is hereby incorporated by reference herein.

The claimed ribozymes are also useful as diagnostic tools to specifically or non-specifically

detect the presence of a target RNA in a sample. That is, the target RNA, if present in the sample, will be specifically cleaved by the ribozyme, and thus can be readily and specifically detected as smaller RNA species. The presence of such smaller RNA species is indicative of the presence of the target RNA in the sample.

Other embodiments are within the following claims.

Claims

1. An enzymatic RNA molecule which cleaves mRNA associated with development or maintenance of chronic myelogenous leukemia, promyelocytic leukemia, Burkitt's lymphoma or acute lymphocytic leukemia, follicular lymphoma, B-cell acute lymphocytic leukemia, breast cancer, colon carcinoma, neuroblastoma, and lung cancer, or which is active to specifically cleave mRNA expressed from a gene encoding multiple drug resistance.

2. The enzymatic RNA molecule of claim 1 which cleaves mRNA produced from the genes PML-RARA, C-myc, bcl-2, E2A-PRL, ErbB2/neu, ras, DCC, N-myc, L-myc or mdr-1.

3. The enzymatic RNA molecule of claim 1, which cleaves target mRNA having a sequence selected from SEQ. ID. NOS. 1-9 in Fig. 2; SEQ. ID. NOS. 1-19 in Fig. 3; SEQ. ID. NOS. 1-62 in Fig. 4; SEQ. ID. NOS. 1-41 in Fig. 5; SEQ. ID. NOS. 1-22 in Fig. 6; SEQ. ID. NOS. 1-71 in Fig. 7; SEQ. ID. NOS. 1-118 in Fig. 8; SEQ. ID. NOS. 1-26 in Fig. 9; SEQ. ID. NOS. 1-66 in Fig. 10; and SEQ. ID. NOS. 1-17 in Fig. 11.

4. The enzymatic RNA molecule of claims 1, 2 or 3, wherein said RNA molecule is in a hammerhead motif.

5. The enzymatic RNA molecule of claim 4, wherein said RNA molecule is in a hairpin, hepatitis Delta virus, group 1 intron, or RNaseP RNA motif.

6. The enzymatic RNA molecule of claim 4, wherein said ribozyme comprises between 5 and 23 bases complementary to said mRNA.

7. The enzymatic RNA molecule of claim 6, wherein said ribozyme comprises between 10 and 18 bases complementary to said mRNA.

8. A mammalian cell including an enzymatic RNA molecule of claims 1, 2 or 3.

9. The cell of claim 8, wherein said cell is a human cell.

10. An expression vector including nucleic acid encoding the enzymatic RNA molecule of claims 1, 2 or 3, in a manner which allows expression of that enzymatic RNA molecule within a mammalian cell.

11. A method for treatment of a disease caused by expression of an mdr-1 gene, chronic myelogenous leukemia, promyelocytic leukemia, Burkitt's lymphoma or acute lymphocytic leukemia, follicular lymphoma, B-cell acute lymphocytic leukemia, breast cancer, colon carcinoma, neuroblastoma, and lung cancer by administering to a patient an enzymatic RNA molecule of claims 1, 2 or 3.

12. The method of claim 11, wherein said patient is a human.

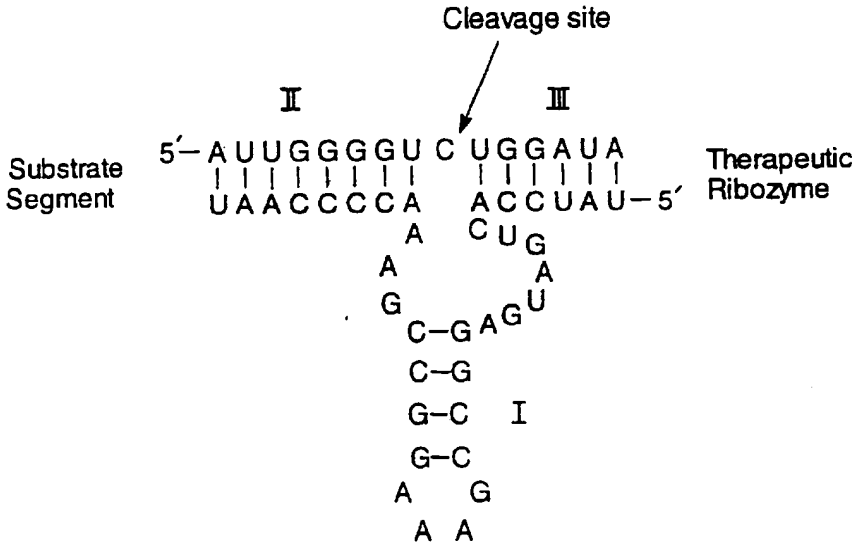
Cleavage site
II
III
Substrate Segment
5′–AUUGGGGUC UGGAUA
UAACCCCA ACCUAU–5′
Therapeutic Ribozyme
A CUG
A A
G U
C–G AG
C–G
G–C I
G–C
A G
A A

FIG. 1

FIG. 2 (MDR-1 Gene)

NUCLEOTIDE NUMBER	SEQUENCE	
NT303	UCUUCCAAGCUCAAAGAAGCAGA	SEQ.ID.NO. 1
NT476	AAACUGAACAAUAAAAGUGAA	SEQ.ID.NO. 2
NT497	AAAGAUAAGAAGGAAAAG	SEQ.ID.NO. 3
NT514	GAAACCAACUGUCAGUGUA	SEQ.ID.NO. 4
NT546	GCUAUUCAAAUUGGCUUGACAA	SEQ.ID.NO. 5
NT585	GAACUUUGGC	SEQ.ID.NO. 6
NT612	CUGGACUUCC	SEQ.ID.NO. 7
NT641	GGAGAAAUGAC	SEQ.ID.NO. 8
NT686	CUGAUGUCAAACAUCACUAAUA	SEQ.ID.NO. 9

FIG. 3 (CML)

Nucleotide Base Reference Number	mRNA Target Sites	Seq. ID No.
bcr		
3135	CUGAUCUCCUCUGACUAUG	01
3196	GUGUUUCAGAAGCUU	02
3214	CCUGACAUCCGUGG	03
3226	GGAGCUGCAGAUGCUGACCA	04
3245	CAACUCGUGUGUGAAACUCC	05
3264	CAGACUGUCCACAGCAUUCCGCUGAC	06
3282	CCGCUGACCAUCAAUAAGG	07
3333	CUGAAUGUCAUCGUCCA	08
3360	GAUUUAAGCAGAGUUCAA	09
abl		
445	AGCCCUUCAGC	10
459	CAGUAGCAUCUG	11
488	CUGAGUGAA	12
542	GAAAAUGACCCC	13
554	AACCUUUUCGUUGC	14
571	GUAUGAUUUUGUG	15
592	AGAUAACACUCUAAGC	16
607	CAUAACUAAA	17
628	CCGGGUCUUAGGCUAUAAUCAC	18
647	CACAAUGGGGAAUGG	19

These targets are present in the 425 nucleotides surrounding the fusion sites of the bcr and abl sequences in the b2a2 and b3a2 recombinant mRNAs. Other sequences in the 5' portion of the bcr mRNA or the 3' portion of the abl mRNA may also be targeted for ribozyme cleavage. Cleavage at any of these sites in the fusion mRNA molecules will result in inhibition of translation of the fusion protein in treated cells.

FIG. 4 (PML)

Nucleotide Number	Sequence	SEQ.ID.NO.
1	CUCCCCUUCAGCUUCUCUUCACGCACUCCAAGAUCUAA	ID.NO.01
78	ACCCGCCCGAUCUCCGAGGCCCCA	ID.NO.02
123	GGAGCCCACCAUGCCUCCCCCCGAGACCC	ID.NO.03
179	GCCCCAGCCCUACAGAGCGAGCCCCCGCU	ID.NO.04
251	CGGAAGCCAAGUGCCCGAAGCUGCUGCCU	ID.NO.05
289	ACGCUGUGCUCAGGA	ID.NO.06
346	CCCUGGCCCCUAG	ID.NO.07
380	AUAACGUCUUUUUCGAGAGUC	ID.NO.08
464	AAGAGUCGGCCGACUUCUGGUGCUUUGAGUGCGAG	ID.NO.09
522	CGAGGCACACCAGUGGUUCCUC	ID.NO.10
550	GAGGCCCGGCCCCUAGCAGAGCUGCGCAACCAGUC	ID.NO.11
591	UGAGUUCCUGGACGGCACCCGCAAGAC	ID.NO.12
618	CAACAACAUCUUCUGCUCCAACCCCAACCAC	ID.NO.13
655	CCUACGCUGACCAGCAUCUACUGCCGAGGAUGUUCCAAG	ID.NO.14
707	GUGCGCGCUCCUUGAC	ID.NO.15
737	AGCUCAAGUGCGACAUCAGCGCAGAGA	ID.NO.16
789	CGCCAUGACGCAGGCGCUGCAGGA	ID.NO.17
885	CGAGACCGAGGAGCUGAUCCGCGA	ID.NO.18
953	AGCUGCUGGAGGCUGUGGA	ID.NO.19
992	ACGAGGAGAUGGCC	ID.NO.20
1025	AUGCUGUGCUGCAGCGCAU	ID.NO.21
1069	AGGAUGAAGUGCUA	ID.NO.22
1102	CUGGACAUGCACGGUUUC	ID.NO.23
1186	GAUGGCUUCGACGAGUUCAA	ID.NO.24
1235	UCACCCAGGGGAAAGCCAUUGAGACCCAGA	ID.NO.25
1277	AAGAGAUAGUGCCCA	ID.NO.26
1408	CGCCGCAGCAUCCA	ID.NO.27
1428	CAUGGUGUACACGU	ID.NO.28
1448	GGGACAAGAACUGCAUCAUCAACAA	ID.NO.29
1492	CAGUACUGCCGACUGCAGAAGUGCUUUGAAGUGGGC	ID.NO.30
1540	UCUGUGAGAAACGACCGAAACAAGAAGAA	ID.NO.31
1569	GAAGGAGGUGCCCAAGCCCGAGUGCUCU	ID.NO.32
1630	CUCAUUGAGAA	ID.NO.33
1690	AAAUACACUACGAACAACA	ID.NO.34
1735	AUUGACCUCUGGGACAA	ID.NO.35
1765	UCCACCAAGUGCAUCAUUAAGACUGUG	ID.NO.36
800	CAAGCAGCUGCCCGGCUUCACCACCCUCA	ID.NO.37
1829	CCAUCGCCGACCAGAUCACC	ID.NO.38

1854	CAAGGCUGCCUGCC	ID.NO.39
1894	ACGCGGUACACGC	ID.NO.40
1934	ACGGGCUGACCCUGAACCGGA	ID.NO.41
1955	CCCAGAUGCACAACGCUGGCUU	ID.NO.42
2018	UGCUGCCCCUGG	ID.NO.43
2036	AUGAUGCGGAGACGGGGCUGCUCAGCG	ID.NO.44
2090	AGGACCUGGAGCAGCCGGACCG	ID.NO.45
2161	CGGAAGCGGAGGCCCAGCCGCCCCCA	ID.NO.46
2192	UCCCCAAGAUGCUAAUGAAGAUUACU	ID.NO.47
2356	GGUGGGGGGCGGGACGG	ID.NO.48
2391	GCCAGGCAGCUGUAGCCCCAGCCUCAGCC	ID.NO.49
2420	CCAGCUCCAACAGAAGCAGCCC	ID.NO.50
2450	ACUCCCCGUGACCGCCCACGCCACAUGGACA	ID.NO.51
2481	CAGCCCUCGCCCUCCG	ID.NO.52
2526	CAUGUGACCCCGCACCAG	ID.NO.53
2572	UACUGGGGACCUUCCC	ID.NO.54
2600	AGGGAGGAGGCAGCGACUCCUUGGAC	ID.NO.55
2657	CCCACAGCCUGGGCUGACGUCAGA	ID.NO.56
2690	CAGGAACUGAG	ID.NO.57
2761	CCCUCUGCCCAGCUCACCACAUCUUCAUCACCAG	ID.NO.58
2824	CAGAACUCACAAGCCAUUGCUC	ID.NO.59
2864	ACCUCCCCCCU	ID.NO.60
2953	AUUAAUUCUCGCUGGUUUUGUUUUUAUUUUAA	ID.NO.61
2995	UUGAUUUUUUUAA	ID.NO.62

FIG. 5 (c-Myc)

nucleotide	mRNA target sequence	SEQ. ID. NO.
(469)	CAGGACCCGCUUCUCUGAAAGGCUCUCCUU	ID.NO. 1
(553)	GCGACGAUGCCCCUCAACGUUAGCU	ID.NO. 2
(589)	AACUAUGACCUCGACUACGACUCGGU	ID.NO. 3
(629)	ACUGCGACGAGGAGGAGAACUUCUACCA	ID.NO. 4
(662)	AGCAGCAGAGCGA	ID.NO. 5
(680)	AGCCCCCGGCGCCCAGCGAGGAUAUCUGGA	ID.NO. 6
(725)	UGCCCACCCCGCC	ID.NO. 7
(758)	CCGGGCUCUGCUCGCCCUCCUA	ID.NO. 8
(783)	UGCGGUCACACCCU	ID.NO. 9
(813)	CAACGACGGCGGUGGCGGGAGCUUCUCCACGGCCGACCA	ID.NO.10
(876)	GGGAGGAGACAU	ID.NO.11
(907)	UGCGACCCGGACGACGAGACCUUCAUCAAAAACAUCAUCA	ID.NO.12
(981)	CGCCAAGCUCGUCUCAGAGAAGCUGGCCUCCUACCA	ID.NO.13
(1023)	GCGCAAAGACAGCG	ID.NO.14
(1043)	CGAACCCCGCCC	ID.NO.15
(1077)	CUCCAGCUUGUACCUGCAGGA	ID.NO.16
(1114)	UCAGAGUGCAUCGACCC	ID.NO.17
(1124)	UCGACCCCUCGGUGGUCUUCCCCUACCCUCUCAACGAC	ID.NO.18
(1168)	UCGCCCAAGUCCUGCGCCUCGCAAGACUCCAGCGC	ID.NO.19
(1230)	CUCCUCGACGGA	ID.NO.20
(1258)	AGCCCCGAGCCCC	ID.NO.21
(1276)	CUCCAUGAGGAGA	ID.NO.22
(1357)	GUGGAAAAGAGG	ID.NO.23
(1376)	CUGGCAAAAGGUCA	ID.NO.24
(1397)	GAUCACCUUCUGCUGGAGGCCACAGCAAACCUCCUCACA	ID.NO.25
(1459)	CACGUCUCCACACAUCAGCACAACUACGCA	ID.NO.26
(1496)	CCUCCACUCGGAAGGACUAUCC	ID.NO.27
(1523)	CCAAGAGGGUCAAGUUGGA	ID.NO.28
(1547)	UCAGAGUCCUGAGACA	ID.NO.29
(1569)	CAACAACCGAAAAUGCA	ID.NO.30
(1643)	UGGAGCGCCAGAGG	ID.NO.31
(1662)	CGAGCUAAAACGGAGCU	ID.NO.32
(1684)	GCCCUGCGUGACCAGAUCCCGGA	ID.NO.33
(1712)	AAAACAAUGAAAAGGCCCCCAAGGUAGUUAUCCUUAAAA	ID.NO.34
(1755)	CACAGCAUACAUCCUGUCCGUCCAAG	ID.NO.35
(1786)	GAGCAAAAGCUCAU	ID.NO.36
(1805)	AAGAGGACUUGUUGCGGAAACGACGAGAACAGUUGAAAC	ID.NO.37
(1845)	CAAACUUGAACAGCUACGGAACUCUUGUGCGU	ID.NO.38
(1883)	AAGUAAGGAAAACGA	ID.NO.39

(1904) CUAACAGAAAUGUCCUGAGCAAUCACCUAUGAACU ID.NO.40

(1964) ACCUCACAACCU ID.NO.41

FIG. 6 (BCL-2)

NUCLEOTIDE	mRNA TARGET SEQUENCE	SEQ.ID.NO.
(1359)	AAAUAGCUGGAUUAUAACU	ID.NO. 01
(1415)	GGCGAGAGGUGCCGUUGGCCCCCGUUGCUUUUCCUCU	ID.NO. 02
(1504)	AUGAAGUACAUC	ID.NO. 03
(1522)	AAGCUGUCGCAGAGGGGCUACGA	ID.NO. 04
(1592)	CACCGGGCAUCUUCUCCUCCCAGCCCGGGCACA	ID.NO. 05
(1656)	CGCCAGGACCUCGCCGCUGCAG	ID.NO. 06
(1719)	CCCGGUGCCACCUGUG	ID.NO. 07
(1729)	CCUGUGGUCCACCUGGCCCUCCGCCAAG	ID.NO. 08
(1683)	GGCUGCCCCCGGC	ID.NO. 09
(1742)	UGGCCCUCCGCCAAGCC	ID.NO. 10
(1807)	AGCCAGCUGCACCUGACGC	ID.NO. 11
(1836)	GCGGGGACGCUUUGCCACG	ID.NO. 12
(1937)	AGAGCGUCAAC	ID.NO. 13
(1972)	AACAUCGCCCUGUGGAUGA	ID.NO. 14
(1997)	ACCUGAACCGGCACCUGCACACCUGGAUCCAGGAUA	ID.NO. 15
(2043)	GGAUGCCUUUGUGGAACUGUACGGCCCCAGCAUG	ID.NO. 16
(2081)	CUCUGUUUGAUUUCUCCUGGCUGUCUC	ID.NO. 17
(2100)	CUGUCUCUGAAGACUCU	ID.NO. 18
(2135)	UGGGAGCUUGCAUCACC	ID.NO. 19
(2180)	GUCAACAUGCCUGCCCCAAACAAAUAUGCAAAAGG	ID.NO. 20
(2235)	AAUAUGCAUUGUCAGUGAUGUACCAUGAAACAAA	ID.NO. 21
(2262)	AAACAAAGCUGCAGGCU	ID.NO. 22

FIG. 7 (B-cell)

Nucleotide Number	Sequence	SEQ.ID.NO.
25	CAGCAGGGUUUCCAGGCCUGA	ID.NO.01
68	GAAUGAACCAGC	ID.NO.02
110	AGGAGCUCAGUGACCUCCUGGACUUCAGCAUGAUG	ID.NO.03
151	CUGCCUGUCACCAA	ID.NO.04
187	CUGGCCGGGGCGCAGUUC	ID.NO.05
211	UCAGGUCUUGAGGA	ID.NO.06
297	CGAGGGCACCCACUUCA	ID.NO.07
319	UCGCACAGCAGCCUCUCUUCAUCCACAUUCCUGGGA	ID.NO.08
425	GCCUGACUCAGGCUGGCUUCCUGUCAGG	ID.NO.09
453	CGAGCUGGCCCUCAACAGCCCCGGGCCCCU	ID.NO.10
507	CUCCCAGUACUACCCCUCCUACUCCGGCA	ID.NO.11
536	GCUCCCGGCGGAGAGCGGCAG	ID.NO.12
557	ACGGCAGCCUAGACACGCAGCCCAAGAAGGUCCGGAAG	ID.NO.13
610	CCAUCCUCGGUGUACCCACCCAGCUCAGGUG	ID.NO.14
701	CCGCCCCCUUCUACGUGGCA	ID.NO.15
726	CAGCCUGCACCC	ID.NO.16
814	CUCCCGCCCGGUAGCGGCCCGGU	ID.NO.17
844	AGUGGAAGCAGCAGCAC	ID.NO.18
866	GUGGCCUGCACCAGCACGAGCGUAUGGGCUA	ID.NO.19
904	CAUGGAGCAGAGGUGAACGGUGG	ID.NO.20
927	GCUCCCAUCUGCAUCCUCCUUCUCCUCAGCCC	ID.NO.21
957	CCGGAGCCACGUAC	ID.NO.22
1021	CUGGGCUCCCGAGGGACCA	ID.NO.23
1047	CAGCUCCGGGGAUGCCCUCGGCAAAGCACUGGCCUC	ID.NO.24
1108	AAUAACUUCUCGUCC	ID.NO.25
1153	GGCCUGGCAGGAACGUCACA	ID.NO.26
1196	GUGCCUUAUCGCCCAGCUACGA	ID.NO.27
1224	UCUCCACGGCCUGCAGAGUAAGAUAGAAGACCACCUGGA	ID.NO.28
1272	CCACGUGCUCC	ID.NO.29
1325	UGCCUGGCCACGGGGC	ID.NO.30
1346	CCUCAGGUUUCA	ID.NO.31
1377	CGGGCGGCACGCAGGC	ID.NO.32
1416	GGACGGCCUCGCAGGCAGCACCAGCCUCAUGCACAAC	ID.NO.33
1453	CACGCGGCCCUCC	ID.NO.34
1474	CCAGGCACCCUCCCCUGACCUGUCUCG	ID.NO.35
1506	CGACUCCUACAGUGUUUUGAGUAUCCGA	ID.NO.36
1551	ACCCACAGACCCCCAGCUGAUGC	ID.NO.37
1606	GCGGGGCCUGAGAAGGGCGGA	ID.NO.38

1632	GGCGGCAGCGGCGGCAGCGGCGGCGGCUUCUG	ID.NO.39
1677	AGACAACUCAGUGGAGCAUUCAGAUUACA	ID.NO.40
1719	ACAGAUCAGACAAAUCUACCAUACG	ID.NO.41
1744	GAGCUGGAGAAAUACGAGCAGGCCUGCAA	ID.NO.42
1773	CGAGUUCACCACCCACGUGAUGAAUCUCCUGCGAG	ID.NO.43
1845	UGAGCGGAUGGUCAGCAUCAUCCACCGCAA	ID.NO.44
1892	UGCAGCUCAAGCAGAGCACGUGC	ID.NO.45
1926	GAUCCUGCGUUCCCGAUUUCUGGAUGCGCGGCG	ID.NO.46
1972	UUCAACAAGCAAGCGAC	ID.NO.47
1995	CCUGAAUGAAU	ID.NO.48
2017	CAUCUCAGCAAC	ID.NO.49
2045	AAGCCAAAGAGGAGUUAGCCAAGAAGUGUGGC	ID.NO.50
2088	CCAGGUAUCAAACUGG	ID.NO.51
2135	ACAUAGGUAAAUUUCAAGAGGAAG	ID.NO.52
2159	CCAAUAUUUAUGCUGCCAA	ID.NO.53
2178	AACAGCUGUCACUGCUACCAAUGU	ID.NO.54
2209	CAUGGAAGCCAAGCUAACUCGCCCUCAACUCCCAACUC	ID.NO.55
2259	CAGUUCUUUUAACAUGUCAAACU	ID.NO.56
2295	CAUGAGCGUGCAGUCACUCAAUGGGGAUUCUUACCAA	ID.NO.57
2347	GCCAACGUGCA	ID.NO.58
2381	AUGUUAUCAGCCAGACAGGAGGAUACA	ID.NO.59
2418	CGCAGCCAGUCAGAUGUAC	ID.NO.60
2442	GCAGGGCAUCAGUGCUAAU	ID.NO.61
2468	GGCAGGAUGCUACUACCCCUUCAUCAGU	ID.NO.62
2503	CCUACAGAAGGCCCUGGCA	ID.NO.63
2546	GAUCUCCCAGCAAUCGCAUCCCGG	ID.NO.64
2638	ACUGGAGGUCGAAGCAAUC	ID.NO.65
2695	GGGAUGCUAUUUCAGCCAAUCU	ID.NO.66
2722	CUUCUUUAUACUCUCUUCCCUUUUUUUUCU	ID.NO.67
2758	AAGCCACCCUUCC	ID.NO.68
2777	CCAGCUGUCAGCCUGGUUUUCGUCAUCUUCC	ID.NO.69
2808	CUGCCCCUGUGCCUC	ID.NO.70
2900	CAAAAAAAUUACAAAGAAAAUAAUAAAA	ID.NO.71

FIG. 8 (Breast Cancer)

Nucleotide	mRNA target sequence	SEQ.ID.NO.
(67)	CCUUUACUGCGCCGCGCGC	ID.NO. 01
(94)	CACCCCUCGCAGCACCCCGC	ID.NO. 02
(113)	GCCCCGCGCCCUCCCAGCCGGGUCCAGCCGGAGCCA	ID.NO. 03
(159)	AGCCGCAGUGAGCACCAUGGAGCU	ID.NO. 04
(240)	CACCCAAGUGUGCACCGGCACAGACAUGAAGCU	ID.NO. 05
(285)	CAGUCCCGAGAC	ID.NO. 06
(305)	ACAUGCUCCGCCACCUCUACCAGGGCUG	ID.NO. 07
(369)	GCCCACCAAUGCCAGCCUGUC	ID.NO. 08
(413)	UGCAGGGCUACGU	ID.NO. 09
(449)	GGCAGGUCCCACUG	ID.NO. 10
(468)	GCUGCGGAUUGUGCGAGGCACCCAGCU	ID.NO. 11
(500)	AGGACAACUAUGCCCUGGCCGUGCUAGACAAUGGAGAC	ID.NO. 12
(580)	GGCCUGCGGGAGCUGCAGCUUCGAAGCCUCACAGAGA	ID.NO. 13
(714)	UCUCACACUGAUAGACACCAACCGCUCUCGGGCCUGC	ID.NO. 14
(747)	CUGCCACCCCUGUUCUCCGAUGUGUAAGGG	ID.NO. 15
(794)	AGAGUUCUGAGGAUUGUCAGA	ID.NO. 16
(849)	CCGCUGCAAGGGGCCACUGCCCACUG	ID.NO. 17
(893)	GUGCUGCCGGCU	ID.NO. 18
(916)	AAGCACUCUGACUGCCUGGCCUGCCUCCACUUCAAC	ID.NO. 19
(951)	CACAGUGGCAUC	ID.NO. 20
(969)	GCUGCACUGCCCAGCC	ID.NO. 21
(990)	CACCUACAACACAGACACGUUUGAG	ID.NO. 22
(1025)	AUCCCGAGGGCCGGUA	ID.NO. 23
(1075)	UACAACUACCUUUCUACGGACGU	ID.NO. 24
(1103)	CCUGCACCCUCGUCUGCCCCCUGCACAACC	ID.NO. 25
(1132)	AAGAGGUGACAGCAGAGGAUGGA	ID.NO. 26
(1218)	GCACUUGCGAG	ID.NO. 27
(1235)	GGGCAGUUACCAGUGCCAAUAU	ID.NO. 28
(1262)	AGUUUGCUGGCUGCAAGAAGAUCUUUG	ID.NO. 29
(1294)	CUGGCAUUUCUGCCGGAGAGCUUUGAUGGGGAC	ID.NO. 30
(1327)	CCAGCCUCCAACACUGCCCC	ID.NO. 31
(1331)	CCUCCAACACUGCCCC	ID.NO. 32
(1357)	GAGCAGCUCCAAGUGUUUGAGAC	ID.NO. 33
(1392)	CACAGGUUACCUAUAC	ID.NO. 34
(1413)	AGCAUGGCCGGACAGCCUGCCUGACCUCAGCGUC	ID.NO. 35
(1454)	ACCUGCAAGUAAUC	ID.NO. 36
(1483)	CACAAUGGCGCCUACUC	ID.NO. 37
(1505)	CCCUGCAAGGGCUGGGCA	ID.NO. 38

(1538)	UGCGCUCACUGAGGGAA	ID.NO. 39
(1560)	CAGUGGACUGGCCCUCAUCCACCAUAACAC	ID.NO. 40
(1590)	CCACCUCUGCUUCGUGCACACGGUGCCCUGGGA	ID.NO. 41
(1622)	CCAGCUCUUUCGGAACC	ID.NO. 42
(1662)	UGCCAACCGGCCAGAGGACGA	ID.NO. 43
(1699)	CUGGCCUGCCACCAGCU	ID.NO. 44
(1749)	CACCCAGUGUGUCAACUGC	ID.NO. 45
(1782)	GGGCCAGGAGUGCG	ID.NO. 46
(1804)	UGCCGAGUACUGCA	ID.NO. 47
(1832)	AGUAUGUGAAUGCCA	ID.NO. 48
(1892)	CAGUGACCUGUUUUGGACC	ID.NO. 49
(1916)	CUGACCAGUGUGUGGCCUGUGCCCACUAUAAGGAC	ID.NO. 50
(1986)	GAAACCUGACCUCUCCUACAUGCCAU	ID.NO. 51
(2033)	AGGGCGCAUGCCAG	ID.NO. 52
(2055)	CAUCAACUGCACCCACUCCUGUGUGGA	ID.NO. 53
(2123)	CUCUGACGUCCAUCAUCUCUGCGGU	ID.NO. 54
(2163)	GGUCGUGGUCUUG	ID.NO. 55
(2212)	AAGAUCCGGAAGUACACGAUGCGGAGA	ID.NO. 56
(2249)	AGGAAACGGAGC	ID.NO. 57
(2286)	AGCGAUGCCCAA	ID.NO. 58
(2303)	CGCAGAUGCGGAUCCUGAAAGAGACGGAGCUGAGGAAGG	ID.NO. 59
(2375)	UCUACAAGGGCAUCUGGA	ID.NO. 60
(2405)	AGAAUGUGAAAAUUCCAG	ID.NO. 61
(2432)	AAGUGUUGAGGGAAAACACAUCCCCCAAAGCCAA	ID.NO. 62
(2466)	CAAAGAAAUCUUAGACGAAGCAUACG	ID.NO. 63
(2516)	AUGUCUCCCGCCUUCUGGGCA	ID.NO. 64
(2542)	CUGACAUCCACGGUGCAGCUGGUGACAC	ID.NO. 65
(2576)	UGCCCUAUGGCUGCCUCUUAGACCAU	ID.NO. 66
(2630)	CCCAGGACCUGCUGAACUGGUGUAU	ID.NO. 67
(2660)	UUGCCAAGGGGA	ID.NO. 68
(2678)	ACCUGGAGGAUGUGC	ID.NO. 69
(2716)	GCUCGGAACGUGCU	ID.NO. 70
(2735)	AGAGUCCCAACCAUGU	ID.NO. 71
(2675)	GCUACCUGGAGGAUGU	ID.NO. 72
(2716)	GCUCGGAACGUGCU	ID.NO. 73
(2737)	AGUCCCAACCAUGUCAAAAUUACAGACUUC	ID.NO. 74
(2774)	CUCGGCUGCUGGACAUUGACGAGACAGAGUACCAUGCAG	ID.NO. 75
(2833)	AAGUGGAUGGCG	ID.NO. 76
(2850)	GUCCAUUCUCCGCCG	ID.NO. 77
(2870)	UCACCCACCAGAGUGAUGUGUGGAGUUAUGGUGUG	ID.NO. 78

(2910)	GUGGGAGCUGAUGACU	ID.NO. 79
(2931)	GGCCAAACCUUAC	ID.NO. 80
(2972)	ACCUGCUGGAAAAGGGGGAGCG	ID.NO. 81
(2990)	AGCGGCUGCCCCAGC	ID.NO. 82
(3010)	AUCUGCACCAUUGAUGUCUACAUGAUCAU	ID.NO. 83
(3064)	GAAUGUCGGCCAAGAUUCCG	ID.NO. 84
(3121)	CCCCAGCGCUUUGUGGUCAUCCAGAAUGAGGACUUGGGC	ID.NO. 85
(3173)	UGGACAGCACCUUCU	ID.NO. 86
(3193)	UCACUGCUGGAGGAC	ID.NO. 87
(3213)	CAUGGGGGACCUGGUGGA	ID.NO. 88
(3236)	AGGAGUAUCUGGUACCC	ID.NO. 89
(3258)	GGGCUUCUUCUGUCCAGACCC	ID.NO. 90
(3299)	UGGUCCACCACAGGCACCGCAGC	ID.NO. 91
(3327)	UACCAGGAGUG	ID.NO. 92
(3352)	ACACUAGGGCUGGAGCCCUCUGAAGAGGAGGCCCC	ID.NO. 93
(3416)	CUGGCUCCGAU	ID.NO. 94
(3434)	AUGGUGACCUGGGAA	ID.NO. 95
(3456)	AGCCAAGGGGCUGCAAAGCCUCCCCACACAUGACCCC	ID.NO. 96
(3493)	AGCCCUCUACAGC	ID.NO. 97
(3511)	AGUGAGGACCCCACA	ID.NO. 98
(3531)	CCUGCCCUCUGAGACUGAUGGCUACGUUGCCCCCCUGA	ID.NO. 99
(3582)	GCCUGAAUAUGUGAACCAGC	ID.NO. 100
(3609)	UCGGCCCCAGC	ID.NO. 101
(3628)	CCCCGAGAGGGCCCUCUGCCUGCUGCCCGACCUGCUGG	ID.NO. 102
(3673)	CUGGAAAGGCCCAAGACUCUCU	ID.NO. 103
(3713)	UCGUCAAAGACGUUUUUG	ID.NO. 104
(3758)	AGUACUUGACACCCCAGGGAGGAGCUGCCCCUCAGCCC	ID.NO. 105
(3821)	CCUUCGACAACCUCUAUUA	ID.NO. 106
(3891)	GACACCUACGGC	ID.NO. 107
(3929)	ACGUGCCAGUGUGAA	ID.NO. 108
(3949)	AGGCCAAGUCC	ID.NO. 109
(3986)	AGCAGGGAAGGCCUGA	ID.NO. 110
(4025)	GAGGGCCCUCCGA	ID.NO. 111
(4045)	CAGGGGAACCUGCCAUGCCAGGAACCUGUCCUA	ID.NO. 112
(4180)	CCCAAUGAGACUC	ID.NO. 113
(4230)	UCCUUCCAGAU	ID.NO. 114
(4253)	AAGCCUUAGGGAAGCUGGCCUGAGAGGGGAAGCGGCCC	ID.NO. 115
(4298)	GUGUCUAAGAACAAAAGCGACCCAUUCAGAGACUG	ID.NO. 116
(4338)	GAAACCUAGUACUGCCCCCCAUGAGGAAGGAACAGCAA	ID.NO. 117
(4416)	UUAGUUUUUACUUUUUUUGUUUUGUUUUUUUAAAG	ID.NO. 118

FIG. 9 (Colon Carcinoma)

Nucleotide	Target mRNA sequence	ID. NOS.
(1)	UCCUAGGCGGCG	ID.NO. 01
(18)	GGCGGCGGAGGCAGCAGCG	ID.NO. 02
(42)	GGCAGUGGCGGCGG	ID.NO. 03
(68)	CGGCUCGGCCAGUACUCCCGGCCC	ID.NO. 04
(120)	GCGCAGGCACUGAAGGCGGCGGCGGGGCCAGAGGCUCAG	ID.NO. 05
(203)	AUAAACUUGUGGUAGUUGGAGCUUGUGGCGU	ID.NO. 06
(248)	UGACGAUACAGCUA	ID.NO. 07
(275)	UUGUGGACGAAUAUGAUCCAACAAUA	ID.NO. 08
(307)	UCCUACAGGAAGCAAGUAGUAAUUGAU	ID.NO. 09
(374)	AAGAGGAGUACAGUGCAAUGAGGGA	ID.NO. 10
(435)	AUUUGCCAUAAAAUAAUACUAAAUCAUUUGAA	ID.NO. 11
(466)	GAUAUUCACCAUUAUAGAGAACAAAUUAAA	ID.NO. 12
(511)	GAAGAUGUACCUAUGGUCCUAGUAGGAAAUAAAUGU	ID.NO. 13
(547)	GAUUUGCCUUCUAGAACAGUAG	ID.NO. 14
(602)	AUGGAAUUCCU	ID.NO. 15
(631)	AAGACAAGACAGGG	ID.NO. 16
(675)	AGAAAUUCGAA	ID.NO. 17
(686)	AACAUAAAGAAAAGAUGAGCAAAGAUGGUAAAAAGA	ID.NO. 18
(722)	AGAAAAAGAAGUCAAAGACAAAGUGUGUA	ID.NO. 19
(781)	UAAGGCAUACUAGU	ID.NO. 20
(820)	ACUAAAUUAUUAG	ID.NO. 21
(840)	UUUAGCAUUACCUAA	ID.NO. 22
(863)	CUGCUCCAUGCAGACUGUUAGC	ID.NO. 23
(907)	AAAAUGACAGUGGA	ID.NO. 24
(946)	UAUUCCCAGAGUU	ID.NO. 25
(970)	ACUAGCAAUGCCUGU	ID.NO. 26

FIG. 10 (Neuroblastoma)

Nucleotide Number	mRNA Sequence	SEQ.ID.NO.
1	ACAGUCAUCUGUCUGGACGC	ID. NO. 01
39	GCUCCUGGGAACUGUGUUGGAGCCGAGCAA	ID. NO. 02
84	AAGCGCGCACAGACUGUAGCCAUCCGAGGACA	ID. NO. 03
153	GAAUCGCCUCCGGAUCC	ID. NO. 04
257	GACGGGAUUGCGACG	ID. NO. 05
339	UUUCCCAGAAAAGCCAGUUCCAG	ID. NO. 06
367	AAGGCAUCCUGGCU	ID. NO. 07
387	GACCCGCCCUAA	ID. NO. 08
434	UCUGCGAAAAGAAAUUCCCU	ID. NO. 09
460	UAGAAGAUCUGUCUGUGUUU	ID. NO. 10
535	CCACGGGAAGGAAGCACCCCCGGUAUUAAAACG	ID. NO. 11
577	GAAAGAAGCCCUCAGUCGCCGGC	ID. NO. 12
635	ACCAUGCCGGGCAUGAUCUGCAAGAAC	ID. NO. 13
662	CCAGACCUCGAGUUUGACUCGCUACAGC	ID. NO. 14
690	CCUGCUUCUACCC	ID. NO. 15
708	AAGAUGACUUCUACUUCGGCGGCC	ID. NO. 16
750	GGGAGGACAUCUGGAAGAAGUUUGA	ID. NO. 17
785	ACGCCCCCGCUGUC	ID. NO. 18
847	CACGGAGAUGCUGCUUGAGAACGAGCUGUG	ID. NO. 19
923	GGCCUCACCCCCA	ID. NO. 20
953	GACUGCAUGUGGA	ID. NO. 21
976	CGCCCGCGAGAAGCUGGAGCGCGCCGUGAGC	ID. NO. 22
1049	ACCGCCCAGUCCCCG	ID. NO. 23
1074	CCGCCAGCCCUGCGGGUC	ID. NO. 24
1133	GCCCUGCCCGCCGAGCUCGCCCACCC	ID. NO. 25
1159	GGCCGCCGAGUGCGUGGAUC	ID. NO. 26
1191	UCCCCUUUCCCGUGAACAAGCGCGAGCCAGCG	ID. NO. 27
1234	AGCCCCGGCCAGUGCCCCGGCG	ID. NO. 28
1369	CAGUACCUCCGGA	ID. NO. 29
1388	ACCCUGAGCGAUUCAGAAUG	ID. NO. 30
1414	ACAUGAGAGUAACUAG	ID. NO. 31
1441	CAGAUGAUGAAGAUGAUGA	ID. NO. 32
1471	AAGAGGAAGAAAUCGAC	ID. NO. 33
1510	GUUCCUCCUCCAACACCAA	ID. NO. 34
1534	UCACCACAUUCACCAUCACUGUGCGUCCCAAGA	ID. NO. 35
1609	UCCUCAAACGAU	ID. NO. 36
1629	AUCCACCAGCAGCACAACUAUGCCGCCCC	ID. NO. 37

1744	CCCCAAAGGCUAAGAGCU	ID. NO. 39
1767	CCCCGAAACUCUGACU	ID. NO. 40
1789	ACAGUGAGCGUCGCAGAAACCACAACAUCCUGG	ID. NO. 41
1847	GUCCAGCUUUCUCAC	ID. NO. 42
1889	AAAGAAUGAGAAGGCC	ID. NO. 43
1917	AUUUUGAAAAA	ID. NO. 44
1935	GAGUAUGUCCACUCCCUCCAGGCC	ID. NO. 45
2031	CACGCUCGGACUUGCUAGACGCUUCUCAAAA	ID. NO. 46
2055	UCAAAACUGGACAGUCACUGCCACUUUGCACAU	ID. NO. 47
2115	GUGUUGACAUUAAG	ID. NO. 48
2162	GAGUUCGGCUCUG	ID. NO. 49
2222	AGCCUGCAUCCCAGGA	ID. NO. 50
2271	UCCAUGACAGCGCUAAACGUU	ID. NO. 51
2297	CGGUUGGGAGCCUCU	ID. NO. 52
2321	GAAGUCACCUUGUGUGUUCCAAGUUUCCAAACA	ID. NO. 53
2376	UAAAAUGGUGCUUAAGUUCCAGCAGAUGCCACA	ID. NO. 54
2417	UUGCCAUUUGAU	ID. NO. 55
2435	GGGGAACAUUU	ID. NO. 56
2453	UACCAUUGACACAU	ID. NO. 57
2478	AUACAUCCUGGGUA	ID. NO. 58
2527	AAGUUCAUACCUAAGUACUGU	ID. NO. 59
2670	UUUGAUACUUAUAUU	ID. NO. 60
2713	UGAGUAGAUAUUACUUUAUCACUUUUUGAACUA	ID. NO. 61
2760	AGAAAUUUACUAUAUAUAUAUGCCUUUUUCCUA	ID. NO. 62
2818	UGUUCAUGUUU	ID. NO. 63
2877	AAUGUAUAUAUUU	ID. NO. 64
2916	AUACCUCAUGUUUAUGAAAAU	ID. NO. 65
2942	GCUUAAUUAAA	ID. NO. 66

FIG. 11 (L-myc)

nucleotide number	mRNA target sequence	Seq.ID. Nos.
9	UGCAGCUCGCGCUCCCGCGCCGAUCCCGAGA	ID.NO. 01
61	AGCGAGGGAGGGCG	ID.NO. 02
93	GCUCGUGAGUGC	ID.NO. 03
152	GCUGCCCCGAG	ID.NO. 04
181	GCUCCAGGUGGC	ID.NO. 05
200	UGGAGCGAGGGGAGCGGACAUGGACUACGACUC	ID.NO. 06
249	ACGACUAUGACUGCGGG	ID.NO. 07
266	GAGGAUUUCUACCGCUCCACGGC	ID.NO. 08
289	GCCCAGCGAGGACAUCUGGA	ID.NO. 09
327	CAUCGCCCCCCACGUC	ID.NO. 10
391	UGGUCCCCCGGAGCCGUGGCCCG	ID.NO. 11
414	GAGGGUGCACCGGAGA	ID.NO. 12
445	GGGCCACUCGAAAGGCU	ID.NO. 13
465	AGGAACUACGCCUCCAUCAUACGCC	ID.NO. 14
490	GUGACUGCAUGUGGAGCGGCUUCUCGGCCCGGG	ID.NO. 15
525	AACGGCUGGAGA	ID.NO. 16
557	GCUCCUGGCGCGC	ID.NO. 17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/04573

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 31/70; C07H 19/10; C12N 5/16, 15/63
US CL :514/44; 536/23.1; 435/240.2, 320.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 536/23.1; 435/240.2, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A1, 91/18624 (Scalon) 12 December 1991, see entire document.	1-12
Y	WO, A1, 91/18625 (Scalon) 12 December 1991, see entire document.	1-12
Y	WO, A1, 92/00080 (Reddy, et. al.) 09 January 1992, see entire document.	1-12
Y	Cell, Volume 47, issued 10 October 1986, M. L. Cleary, et. al., "Cloning And Structural Analysis Of cDNAs For bcl-2 And A Hybrid bcl-2/Immunoglobulin Transcript Resulting From The t(14;18) Translocation", pages 19-28, see entire document.	1-12

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 August 1993	17 AUG 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer CHARLES PATTERSON Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/04573

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Volume 247, issued 09 March 1990, N. Sarver, et. al., "Ribozymes As Potential Anti-HIV-1 Therapeutic Agents", pages 1222-1225, see entire document.	1-12
Y	Mol. Cell. Biol., Volume 8, No. 1, issued January 1988, F. Kaye, et. al., "Structure And Expression Of The Human L-myc Gene Reveal A Complex Pattern Of Alternative mRNA Processing", pages 186-195, see entire document.	1-12
Y	Nature, Volume 304, issued 11 August 1983, J. P. McGrath, et. al., "Structure And Organization Of The Human Ki-ras Proto-oncogene And A Related Processed Pseudogene", pages 501-506, see entire document.	1-12
Y	Proc. Natl. Acad. Sci. USA, Volume 82, issued October 1985, K. Semba, et. al., "A v-erbB-Related Protoocogene, C-erB-2, Is Distinct From The c-erbB-1/Epidermal Growth Factor-Receptor Gene And Is Amplified In A Human Salivary Gland Adenocarcinoma", pages 6497-6501, see entire document.	1-12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/04573

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-12, drawn to an enzymatic RNA which cleaves mRNA associated with chronic myelogenous leukemia, a cell containing this RNA, a vector that encodes this RNA, and a method of use in treating a disease.
II. Claims 1-12, drawn to an enzymatic RNA which cleaves mRNA associated with promyelocytic leukemia, a cell containing this RNA, a vector that encodes this RNA, and a method of use in treating a disease.
III. Claims 1-12, drawn to an enzymatic RNA which cleaves mRNA associated with Burkitt's lymphoma, a cell containing this RNA, a vector that encodes this RNA, and a method of use in treating a disease.
IV. Claims 1-12, drawn to an enzymatic RNA which cleaves mRNA associated with acute lyphocytic leukemia, a cell containing this RNA, a vector that encodes this RNA, and a method of use in treating a disease.
V. Claims 1-12, drawn to an enzymatic RNA which cleaves mRNA associated with follicular lymphoma, a cell containing this RNA, a vector that encodes this RNA, and a method of use in treating a disease.
VI. Claims 1-12, drawn to an enzymatic RNA which cleaves mRNA associated with B-cell acute lymphocytic leukemia, a cell containing this RNA, a vector that encodes this RNA, and a method of use in treating a disease.
VII. Claims 1-12, drawn to an enzymatic RNA which cleaves mRNA associated with breast cancer, a cell containing this RNA, a vector that encodes this RNA, and a method of use in treating a disease.
VIII. Claims 1-12, drawn to an enzymatic RNA which cleaves mRNA associated with colon carcinoma, a cell containing this RNA, a vector that encodes this RNA, and a method of use in treating a disease.
IX. Claims 1-12, drawn to an enzymatic RNA which cleaves mRNA associated with neuroblastoma, a cell containing this RNA, a vector that encodes this RNA, and a method of use in treating a disease.
X. Claims 1-12, drawn to an enzymatic RNA which cleaves mRNA associated with lung cancer, a cell containing this RNA, a vector that encodes this RNA, and a method of use in treating a disease.